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(54) Title: MULTIPLY-SUBSTITUTED PROTEASE VARIANTS

(57) Abstract

Novel protease variants derived from the DNA sequences of naturally-occurring or recombinant non-human proteases are disclosed. The variant proteases, in general, are obtained by in vitro modification of a precursor DNA sequence encoding the naturally-occurring or recombinant protease to generate the substitution of a plurality of amino acid residues in the amino acid sequence of a precursor protease. Such variant proteases have properties which are different from those of the precursor protease, such as altered wash performance. The substituted amino acid residue correspond to positions 62, 212, 230, 232, 252 and 257 of Bacillus amyloliquefaciens subtilisin.

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MULTIPLY-SUBSTITUTED PROTEASE VARIANTS

Related Applications

The present application is a continuation-in-part application of United States
Patent Application 08/956,323, filed October 23, 1998. United States Patent
Application 08/956,564, filed October 23, 1998, and United States Patent Application
08/956,324 filed October 23, 1998, all of which are hereby incorporated herein in
their entirety.

10 Background of the Invention

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Serine proteases are a subgroup of carbonyl hydrolases. They comprise a diverse class of enzymes having a wide range of specificities and biological functions. Stroud, R. Sci. Amer., 131:74-88. Despite their functional diversity, the catalytic machinery of serine proteases has been approached by at least two genetically distinct families of enzymes: 1) the subtilisins and 2) the mammalian chymotrypsin-related and homologous bacterial serine proteases (e.g., trypsin and S. gresius trypsin). These two families of serine proteases show remarkably similar mechanisms of catalysis. Kraut, J. (1977), Annu. Rev. Biochem., 46:331-358. Furthermore, although the primary structure is unrelated, the tertiary structure of these two enzyme families bring together a conserved catalytic triad of amino acids consisting of serine, histidine and aspartate.

Subtilisins are serine proteases (approx. MW 27,500) which are secreted in large amounts from a wide variety of *Bacillus* species and other microorganisms. The protein sequence of subtilisin has been determined from at least nine different species of *Bacillus*. Markland, F.S., et al. (1983), Hoppe-Seyler's Z. Physiol. Chem., 364:1537-1540. The three-dimensional crystallographic structure of subtilisins from *Bacillus amyloliquefaciens*, *Bacillus licheniforimis* and several natural variants of *B. lentus* have been reported. These studies indicate that although subtilisin is genetically unrelated to the mammalian serine proteases, it has a similar active site structure. The x-ray crystal structures of subtilisin containing covalently bound peptide inhibitors (Robertus, J.D., et al. (1972), <u>Biochemistry</u>, 11:2439-2449) or product complexes (Robertus, J.D., et al. (1976), <u>J. Biol. Chem.</u>, 251:1097-1103) have also provided information regarding the active site and putative substrate binding cleft of subtilisin. In addition, a large number of kinetic and chemical

modification studies have been reported for subtilisin; Svendsen, B. (1976), Carlsberg Res. Commun., 41:237-291; Markland, F.S. Id.) as well as at least one report wherein the side chain of methionine at residue 222 of subtilisin was converted by hydrogen peroxide to methionine-sulfoxide (Stauffer, D.C., et al. (1965), J. Biol. Chem., 244:5333-5338) and extensive site-specific mutagenesis has been carried out (Wells and Estell (1988) TIBS 13:291-297)

Summary of the invention

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It is an object herein to provide a protease variant containing a substitution of an amino acid at one or more residue positions corresponding to residue positions selected from the group consisting of 62, 212, 230, 232, 252 and 257 of Bacillus amyloliquefaciens subtilisin.

While any combination of the above listed amino acid substitutions may be employed, the preferred protease variant enzymes of the present invention comprise the substitution of amino acid residues in the following combinations. All of the residue positions correspond to positions of Bacillus amyloliquefaciens subtilisin:

- (1) a protease variant including substitutions of the amino acid residues at position 62 and at one or more of the following positions 103, 104, 109, 159, 213, 232, 236, 245, 248 and 252;
- (2) a protease variant including substitutions of the amino acid residues at position 212 and at one or more of the following positions 12, 98, 102, 103, 104, 159, 232, 236, 245, 248 and 252;
- (3) a protease variant including substitutions of the amino acid residues at position 230 and at one or more of the following positions 68, 103, 104, 159. 232, 236 and 245;
- (4) a protease variant including substitutions of the amino acid residues at position 232 and at one or more of the following positions: 1, 9, 12, 61, 62, 68, 76, 97, 98, 101, 102, 103, 104, 109, 130, 131, 159, 183, 185, 205, 209, 210, 212, 213, 217, 230, 236, 245, 248, 252, 257, 260, 270 and 275;
- (5) a protease variant including substitutions of the amino acid residues at position 232 and at on or more of the following positions 103, 104, 236 and 245;
- (6) a protease variant including substitutions of the amino acid residues at position 232 and 103 and at on or more of the following positions 1, 9, 12, 61, 62,

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- 68, 76, 97, 98, 101, 102, 103, 104, 109, 130, 131, 159, 183, 185, 205, 209, 210, 212, 213, 217, 230, 236, 245, 248, 252, 257, 260, 270 and 275:
- (7) a protease variant including substitutions of the amino acid residues at position 232 and 104 and at one or more of the following positions 1, 9, 12, 61, 62, 68, 76, 97, 98, 101, 102, 103, 104, 109, 130, 131, 159, 183, 185, 205, 209, 210, 212, 213, 217, 230, 236, 245, 248, 252, 257, 260, 270 and 275;
- (8) a protease variant including substitutions of the amino acid residues at position 232 and 236 and at one or more of the following positions 1, 9, 12, 61, 62, 68, 76, 97, 98, 101, 102, 103, 104, 109, 130, 131, 159, 183, 185, 205, 209, 210, 212, 213, 217, 230, 236, 245, 248, 252, 257, 260, 270 and 275;
- (9) a protease variant including substitutions of the amino acid residues at position 232 and 245 and at one or more of the following positions 1, 9, 12, 61, 62, 68, 76, 97, 98, 101, 102, 103, 104, 109, 130, 131, 159, 183, 185, 205, 209, 210, 212, 213, 217, 230, 236, 245, 248, 252, 257, 260, 270 and 275;
- (10) a protease variant including substitutions of the amino acid residues at position 232, 103, 104, 236 and 245 and at one or more of the following positions 1, 9, 12, 61, 62, 68, 76, 97, 98, 101, 102, 103, 104, 109, 130, 131, 159, 183, 185, 205, 209, 210, 212, 213, 217, 230, 236, 245, 248, 252, 257, 260, 270 and 275;
 - (11) a protease variant including substitutions of the amino acid residues at position 252 and at one or more of the following positions 1, 9, 12, 61, 62, 68, 97, 98, 101, 102, 103, 104, 109, 130, 131, 159, 183, 185, 210, 212, 213, 217, 232, 236, 245, 248 and 270;
 - (12) a protease variant including substitutions of the amino acid residues at position 252 and at one or more of the following positions 103, 104, 236 and 245;
- (13) a protease variant including substitutions of the amino acid residues at positions 252 and 103 and at one or more of the following positions 1, 9, 12, 61, 62, 68, 97, 98, 101, 102, 103, 104, 109, 130, 131, 159, 183, 185, 210, 212, 213, 217, 232, 236, 245, 248 and 270;
- (14) a protease variant including substitutions of the amino acid residues at positions 252 and 104 and at one or more of the following positions 1, 9, 12, 61, 62, 68, 97, 98, 101, 102, 103, 104, 109, 130, 131, 159, 183, 185, 210, 212, 213, 217, 232, 236, 245, 248 and 270;
 - (15) a protease variant including substitutions of the amino acid residues at positions 252 and 236 and at one or more of the following positions 1, 9, 12, 61, 62.

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68, 97, 98, 101, 102, 103, 104, 109, 130, 131, 159, 183, 185, 210, 212, 213, 217, 232, 236, 245, 248 and 270;

- (16) a protease variant including substitutions of the amino acid residues at positions 252 and 245 and at one or more of the following positions 1, 9, 12, 61, 62, 68, 97, 98, 101, 102, 103, 104, 109, 130, 131, 159, 183, 185, 210, 212, 213, 217, 232, 236, 245, 248 and 270;
- (17) a protease variant including substitutions of the amino acid residues at positions 252, 103, 104, 236 and 245 and at one or more of the following positions 1, 9, 12, 61, 62, 68, 97, 98, 101, 102, 103, 104, 109, 130, 131, 159, 183, 185, 210, 212, 213, 217, 232, 236, 245, 248 and 270; and
- (18) a protease variant including substitutions of the amino acid residues at position 257 and at one or more of the following positions 68, 103, 104, 205, 209, 210, 232, 236, 245 and 275. More preferred protease variants are substitution sets selected from the group consisting of residue positions corresponding to positions in Table 1 of *Bacillus amyloliquefaciens* subtilisin:

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													245		245	252		252
											245		236	252	236	248		245
			252					271	271		236		232	245	232	245	245	236
			217	159	271	271	271	212	243		232		159	236	213	236	236	232
271	261	258	159	104	268	212	245	141	236	104	159	245	104	232	159	232	232	159
212	252	212	104	103	212	104	212	134	212	103	104	232	103	159	104	159	159	140
104	104	104	103	76	104	103	104	104	104	76	103	104	9/	104	103	104	104	104
103	103	103	76	62	103	87	103	103	103	62	76	103	68	103	76	103	103	103
9/	76	76	4	12	92	9/	92	9/	92	20	89	92	24	89	89	89	89	89

			275															
252		252	252							245		245						
245	245	245	245	257	245	248	245	245	245	236	245	236	245	245	245	245	248	245
236	236	236	236	245	236	245	236	236	237	232	236	232	236	236	236	236	245	236
232	232	232	232	236	232	236	232	232	236	159	232	206	232	232	232	232	236	232
159	159	159	159	232	159	232	159	203	232	104	183	174	188	230	159	215	232	159
104	104	104	104	159	116	159	104	159	159	103	159	159	159	159	104	159	159	104
103	103	103	103	5	104	104	103	104	104	62	104	104	104	104	103	104	104	103
68	68	68	87	103	103	103	68	103	103	9/	103	103	103	103	98	103	103	9/
43	43	43	89	89	89	68	10	88	89	89	89	89	89	89	89	68	88	89

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									245			259	260				245	
245	257		275		257		245	245	236	245	245	245	245	261		245	236	
236	245		257		245	257	236	236	232	236	236	236	236	245		236	232	
232	236	257	245		236	245	232	232	214	232	232	232	232	236	245	232	159	
210	232	245	236		232	236	209	211	159	215	159	159	159	232	242	210	104	245
159	159	236	232	275	224	232	159	159	104	159	104	104	104	159	236	159	103	236
104	104	232	159	257	159	159	104	104	103	104	103	103	103	104	232	104	9/	232
103	103	104	104	104	104	104	103	103	76	103	76	76	76	103	104	103	68	<u>4</u>
92	92	103	103	103	103	103	76	9/	89	9/	89	89	28	92	103	92	48	103
89	68	76	89	76	68	76	68	68	12	68	12	20	68	68	76	89	12	76

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							245				252							
	251	272	245				236	252	252	252	248		252	252	252	252	261	252
	248	245	236	256	245	245	232	248	248	248	245		248	248	248	248	252	248
245	245	236	232	245	236	236	185	245	245	245	236	252	245	245	245	245	248	245
236	236	232	206	236	232	232	170	236	236	236	232	248	236	236	236	236	245	236
232	232	159	183	232	206	159	159	232	232	232	184	245	232	232	232	232	236	232
192	159	104	159	159	159	104	116	159	159	212	159	236	209	159	159	209	232	185
159	147	103	\$	104	104	103	104	104	104	159	66	232	159	109	104	159	159	159
104	104	76	103	103	103	78	103	103	103	104	104	159	104	104	103	\$	104	Ş
103	103	68	9/	9/	92	89	9/	89	89	103	103	104	103	103	89	103	103	103
92	9/	12	68	68	68	27	68	61	43	89	89	103	68	68	20	89	89	88

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	252																	269
252	248	252	252		252	252	252	252	252	252		252	255	256	260	257	258	252
248	245	248	248	252	248	248	248	248	251	248	252	248	252	252	252	252	252	248
245	236	245	245	248	245	245	245	245	248	245	248	245	248	248	248	248	248	245
236	232	236	236	245	236	236	236	236	245	236	245	236	245	245	245	245	245	236
232	210	232	232	236	232	232	232	232	236	232	236	232	236	236	236	236	236	232
210	185	212	213	232	215	216	159	173	232	206	232	159	232	232	232	232	232	159
159	159	159	159	213	159	159	104	159	159	159	159	104	159	159	159	159	159	104
104	104	104	104	104	104	104	103	104	104	104	104	103	104	104	104	104	104	103
103	103	103	103	103	103	103	89	103	103	103	103	89	103	103	103	103	103	89
68	68	68	68	68	89	68	20	68	68	68	68	55	68	68	68	68	68	8

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260									245		252	245	252		252	248		
252	261	261	252				252		236	252	248	236	248		248	245	252	
248	252	252	248			252	248	252	232	248	245	232	245		245	236	248	252
245	248	248	245	252	252	248	245	248	218	245	236	213	236	245	236	232	245	248
236	245	245	236	248	248	245	236	245	213	236	232	210	232	236	232	159	236	245
232	236	236	232	245	245	236	232	236	159	232	159	159	159	232	159	137	232	236
159	232	232	159	236	236	232	159	232	104	228	104	104	104	210	130	133	159	232
116	159	159	104	232	232	159	104	159	103	159	103	103	103	205	104	104	133	159
104	104	104	103	104	159	1 0	103	104	101	104	76	89	76	159	103	103	104	104
103	103	103	76	103	\$	103	89	103	76	103	89	92	89	104	89	89	103	103
88	68	89	68	89	103	89	18	68	89	89	33	68	61	103	61	61	61	89

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248	245	245	245	252	252	252	252	252	252	252	261	252	252	252	252	245	248	248
245	236	236	236	248	248	248	248	248	248	248	252	248	248	248	248	236	245	245
236	232	232	232	245	245	245	245	245	245	245	248	245	245	245	245	232	236	236
232	160	104	167	236	236	236	236	236	236	236	245	236	236	236	236	213	232	232
218	159	103	159	232	232	232	232	232	232	232	236	232	232	232	232	159	213	217
159	104	76	104	159	159	159	159	159	159	159	232	159	184	166	217	104	159	206
104	103	68	103	104	104	104	104	104	106	109	159	104	159	159	159	103	104	159
103	89	61	89	103	103	103	103	103	104	104	104	103	104	104	104	62	103	4
68	61	3	61	97	98	66	101	102	103	103	103	62	103	103	103	20	62	103

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252	_		,		260	260	245	245	245	260								
248	252	252	252	252	245	245	236	236	236	245	260	260						
245	248	248	248	248	236	236	232	232	232	236	245	245	245	245	245	245	245	245
236	245	245	245	245	232	232	213	213	213	232	236	236	236	236	236	236	236	236
232	236	236	236	236	213	213	209	210	205	210	232	232	232	232	232	232	232	232
206	232	232	232	232	159	159	159	159	159	159	213	213	209	210	230	126	205	210
159	159	159	159	159	104	104	104	104	104	104	159	159	159	159	159	159	159	159
104	130	131	104	104	103	103	103	103	103	103	104	104	104	104	104	104	104	104
103	104	104	103	103	9/	76	92	9/	9/	9/	103	103	103	103	103	103	103	103
62	103	103	27	38	38	68	68	68	68	68	68	92	89	89	68	68	68	89

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			257	257	257		245	261		252		236	245		245	260	236	245
	260		245	245	245		236	257	260	248	257	232	236		236	245	232	236
	245		236	236	236	257	232	245	245	245	245	213	232	257	232	236	210	232
245	236	245	232	232	232	245	213	236	236	236	236	210	213	245	213	232	209	210
236	232	236	174	194	209	236	159	232	232	232	232	159	209	236	210	209	205	209
230	159	232	159	159	159	232	104	159	213	210	209	104	159	232	205	205	159	205
159	104	159	104	104	104	159	103	104	159	159	159	103	104	209	159	159	104	159
104	103	104	103	103	103	104	9/	103	104	104	104	9/	103	104	104	104	103	104
103	68	103	99	68	68	103	68	68	103	103	103	89	12	103	103	103	68	103

	260										252	252	252					256
	245							252	261	252	248	248	248	252				252
257	236	245					245	248	257	248	245	245	245	248	252	252	252	248
245	232	236	245	245	245		236	245	245	245	236	236	236	245	248	248	248	245
236	210	232	236	236	236	245	232	236	236	236	232	232	232	236	245	245	245	236
232	209	210	232	232	232	236	209	232	232	232	212	212	212	232	236	236	244	232
209	205	209	210	210	159	230	159	159	159	212	159	159	159	213	232	232	236	213
205	159	205	209	205	128	159	104	104	104	159	104	104	104	159	159	184	232	159
159	104	159	159	159	104	104	103	103	103	104	103	103	103	104	131	159	159	104
5	103	104	104	104	103	103	88	89	89	103	102	102	102	103	104	104	104	103
103	89	103	103	103	89	48	48	48	48	102	12	101	86	102	103	103	103	62

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										252								
252						252		252	252	248			252			252	252	252
248	252	252	252	252	252	248	252	248	248	245			248	252	252	248	248	248
245	248	248	248	248	248	245	248	245	245	236			245	248	248	245	245	245
236	245	245	245	245	245	236	236	236	236	232			236	245	245	236	236	236
232	236	236	236	236	236	232	232	232	232	213	252		232	236	236	232	232	232
213	232	232	232	232	232	212	212	213	213	212	248		213	232	232	213	213	213
159	185	206	213	159	159	159	159	159	212	159	245	245	159	159	159	159	159	159
104	159	159	159	104	104	104	104	109	159	104	232	230	130	130	128	104	128	128
103	104	104	104	103	103	103	103	104	104	103	159	159	104	104	104	103	104	104
62	103	103	103	102	102	102	102	103	103	101	104	104	103	103	103	101	103	103
12	101	101	101	86	101	98	98	62	62	62	103	103	62	101	101	62	62	79

				—							—-т	
												271
												252
										252		248
260	252	252	252	252	252	252	252		252	248	252	245
252	248	248	248	248	248	248	248		248	245	248	236
248	245	245	245	245	245	245	245		245	236	245	232
245	236	236	236	236	236	236	236	245	236	232	236	213
236	232	232	232	232	232	232	232	236	232	194	232	206
232	159	159	159	212	209	210	205	230	194	159	230	185
159	131	104	104	159	159	159	159	159	159	104	159	159
104	104	103	103	104	104	104	104	104	104	103	104	104
103	103	101	101.	103	103	103	103	103	103	101	103	103
101	101	98	99	101	101	101	101	101	101	76	101	62

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Most preferred protease variants are substitution sets selected from the group consisting of residue positions corresponding to positions in Table 2 of *Bacillus* amyloliquefaciens subtilisin:

lable 2

N76D	S103A	V104I	S212P	E271V							
N76D	S103A	V104I	N252K	N261Y							
N76D	S103A	V104I	S212P	G258R							
V4E	N76D	S103A	V104I	G159D	L217E	N252D					
Q12H	N62H	N76D	S103A	V104I	G159D						
N76D	S103A	V104I	S212P	V268F	E271V						
N76D	S87R	S103A	V104I	S212P	E271V						
N76D	S103A	V104I	S212P	Q245L	E271V						
N76D	S103A	V104I	T134S	S141N	S212P	E271V					
N76D	S103A	V104I	S212P	Q236L	N243S	E271V					
G20V	N62S	N76D	S103A	V104I							İ
V68A	N76D	S103A	V104I	G159D	A232V	Ф236Н	Q245R				
N76D	S103A	V104I	A232V	Q245R							
S24T	V68A	N76D	S103A	V104I	G159D	A232V	Q236 Н	Q245R			
V68A	S103A	V104I	G159D	A232V	0236Н	Q245R	N252K				
V68A	N76D	S103A	V104I	G159D	T213R	A232V	Q236Н	Q245R	T260A		
V68A	S103A	V104I	G159D	A232V	Ф236Н	Q245R	N248D	N252K			
V68A	S103A	V104I	G159D	A232V	Ф236Н	Q245R					
V68A	S103A	V104I	N140D	G159D	A232V	Q236H	Q245R	N252K			

N43S	V68A	S103A	V104I	G159D	A232V	Ф236Н	Q245R	N252K			
N43K	V68A	S103A	V104I	G159D	A232V	Ф236Н	Q245R				
N43D	V68A	S103A	V104I	G159D	A232V	α236Н	Q245R	N252K			
V68A	S87G	S103A	V104I	G159D	A232V	Q236Н	Q245R	N252K	R275S		
V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	L257V				
V68A	S103A	V104I	N116D	G159D	A232V	Q236Н	Q245R				
V68A	S103A	V104I	G159D	A232V	0236Н	Q245R	N248D				
R10C	V68A	S103A	V104I	G159D	A232V	Q236Н	Q245R				
V68A	S103A	V104I	G159D	V203E	A232V	Q236Н	Q245R				
V68A	S103A	V104I	G159D	A232V	Q236 Н	K237E	Q245R				
V68A	N76D	N621	S103A	V104I	G159D	A232V	О236Н	Q245R			
V68A	S103A	V104I	G159D	N183D	A232V	Q236 Н	Q245R				
V68A	S103A	V104I	G159D	A174V	Q206L	A232V	0236Н	Q245R			
V68A	S103A	V104I	G159D	S188C	A232V	Q236Н	Q245R				
V68A	S103A	V104I	G159D	A230T	A232V	α236Н	Q245R		-		
V68A	A98T	S103A	V104I	G159D	A232V	Q236Н	Q245R				
V68A	S103A	V104I	G159D	A215T	A232V	Q236Н	Q245R				
V68A	S103A	V104I	G159D	A232V	Q236Н	Q245R	N248S			-	
V68A	M76D	S103A	V104I	G159D	A232V	Q236 Н	Q245R				

V68A N7		5000	V104I	CECIO	72127	A232V	UZ30H	U245K			
	N76D	S103A	V104I	G159D	A232V	Q236Н	Q245R	L257V			-
N76D S1	S103A	V104I	A232V	Q236 Н	Q245R	L257V					
V68A S1	S103A	V104I	G159D	A232V	0236Н	Q245R	L257V	R275H			
N76D S1	S103A	V104I	L257V	R275H							
V68A S1	S103A	V104I	G159D	T224A	A232V	Q236 Н	Q245R	L257V			
N76D S1	S103A	V104I	G159D	A232V	0236Н	Q245R	L257V				
V68A N7	N76D	S103A	V104I	G159D	Y209W	A232V	Q236 Н	Q245R			
V68A N7	N76D	S103A	V104I	G159D	G211R	A232V	Q236 Н	Q245R			
V68A N7	N76D	S103A	V104I	G159D	G211V	A232V	Q236 Н	Q245R			
Q12R VE	V68A	N76D	S103A	V104I	G159D	Y214L	A232V	Q236Н	Q245R		
V68A N7	N76D	S103A	V104I	G159D	A215R	A232V	Ф236Н	Q245R			
Q12R V6	V68A	N76D	S103A	V104I	G159D	A232V	Ф236Н	Q245R		_	
G20R V6	V68A	N76D	S103A	V104I	G159D	A232V	Q236 Н	Q245R	S259G	-	
V68A S8	S87R	N76D	S103A	V104I	G159D	A232V	Q236 Н	Q245R	T260V		
V68A N7	N76D	S103A	V104I	G159D	A232V	Q236Н	Q245R	N261G			
V68A N7	N76D	S103A	V104I	G159D	A232V	Q236Н	Q245R	N261W			
N76D S1	S103A	V104I	A232V	Q236Н	S242P	Q245R					
V68A N7	N76D	S103A	V104I	G159D	P210L	A232V	A232V Q236H	Q245R			

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Q12R	A48V	V68A	N76D	S103A	V104I	G159D	A232V	Ф236Н	Q245R		
N76D	S103A	V104I	A232V	Q236 Н	Q245R						
N76D	S103A	V104I	G159D	Y192F	A232V	Q236Н	Q245R				
N76D	S103A	V104I	V147I	G159D	A232V	Q236H	Q245R	N248S	K251R		
Q12R	V68A	N76D	S103A	V104I	G159D	A232V	Ф236Н	Q245R	A272S		
V68A	N76D	S103A	V104I	G159D	N183K	Q206L	A232V	Ф236Н	Q245R		
V68A	N76D	S103A	V104I	G159D	A232V	Q236 Н	Q245R	S256R			
V68A	N76D	S103A	V104I	G159D	Q206R	A232V	Q236 Н	Q245R			
K27R	V68A	N76D	S103A	V104I	G159D	A232V	Q236H	Q245R			
V68A	N76D	S103A	V104I	N116T	G159D	R170S	N185S	A232V	Q236 Н	Q245R	
G61E	V68A	S103A	V104I	G159D	A232V	Q236 Н	Q245R	N248D	N252K		
N43D	V68A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	S212P	A232V	Q236 Н	Q245R	N248D	N252K		
V68A	S103A	V104I	N66S	G159D	N184D	A232V	Ф236Н	Q245R	N248D	N252K	
S103A	V104I	G159D	A232V	Q236Н	Q245R	N248D	N252K				
V68A	S103A	V104I	G159D	Y209W	A232V	Q236 Н	Q245R	N248D	N252K		
V68A	S103A	V104I	Q109R	G159D	A232V	Ф236Н	Q245R	N248D	N252K		
G20R	V68A	S103A	V104I	G159D	A232V	Ф236Н	Q245R	N248D	N252K		
V68A	S103A	V1041	G159D	Y209F	A232V	Ω236Н	Q245R	N248D	N252K		

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V68A	S103A	V104I	G159D	A232V	Ф236Н	Q245R	N248D	N252K	N261D		
V68A	S103A	V104	G159D	N185D	A232V	Ф236Н	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	P210R	A232V	Ф236Н	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	P210T	A232V	Q236 Н	Q245R	N248D	N252K		
V68A	S103A	V1041	G159D	P210S	A232V	Ф236Н	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	N185D	P210L	A232V	Q236H	Q245R	N248D	N252K	
V68A	S103A	V104I	G159D	P210L	A232V	Q236Н	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	S212A	A232V	Q236H	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	S212G	A232V	Ω236Н	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	S212E	A232V	Q236 Н	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	T213E	A232V	Q236 Н	Q245R	N248D	N252K		
V68A	S103A	V104I	T213S	A232V	Q236Н	Q245R	N248D	N252K			
V68A	A103V	V104I	G159D	T213E	A232V	Q236Н	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	T213G	A232V	Q236H	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	A215V	A232V	α236Н	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	A215R	A232V	Ф236Н	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	S216T	A232V	Q236H	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	S216V	A232V	Q236H	Q236H Q245R	N248D	N252K		

V68A	S103A	V104I	G159D	S216C	A232V	Ф236Н	Q245R	N248D	N252K		
G20A	V68A	S103A	V104I	G159D	A232V	Q236Н	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	N173D	A232V	д 236Н	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	A232V	Q236 Н	Q245R	N248D	K251V	N252K		
V68A	S103A	V104I	G159D	Q206R	A232V	Q236 Н	Q245R	N248D	N252K		
V68A	S103A	V104I	G159D	A232V	Ω236Н	Q245R	N248D	N252F			
V68A	S103A	V104I	G159D	A232V	Ω236Н	Q245R	N248D	N252L			
P55S	V68A	S103A	V104I	G159D	A232V	Ф236Н	Q245R	N248D	N252F		
V68A	S103A	V104I	G159D	A232V	Q236 Н	Q245R	N248D	N252K	T255V		
V68A	S103A	V104I	G159D	A232V	Ф236Н	Q245R	N248D	N252K	S256N		
V68A	S103A	V1041	G159D	A232V	Q236 Н	Q245R	N248D	N252K	S256E		
V68A	S103A	V1041	G159D	A232V	Q236 Н	Q245R	N248D	N252K	S256R		
V68A	S103A	V104I	G159D	A232V	Ф236Н	Q245R	N248D	N252K	T260R		<u>.</u>
V68A	S103A	V104I	G159D	A232V	Q236Н	Q245R	N248D	N252K	L257R		
V68A	S103A	V104I	G159D	A232V	Q236 Н	Q245R	N248D	N252K	G258D		
187	V68A	S103A	V104I	G159D	A232V	Q236Н	Q245R	N248D	N252K	N269D	
V68A	S103A	V104I	N116S	G159D	A232V	Q236 Н	Q245R	N248D	N252K	T260E	
V68A	S103A	V104I	G159D	A232V	Q236Н	Q245R	N248D	N252K	N261R		
V68A	S103A	V104I	G159D	A232V	Q236 Н	Q236H Q245R	N248D	N252K	N261D		

V68A	N76D	S103A	V104I	G159D	A232V	Q236 Н	Q245R	N248D	N252K			
V68A	S103A	V104I	A232V	Ф236Н	Q245R	N248D	N252K					
S103A	V104I	G159D	A232S	Q236Н	Q245R	N248D	N252K					
V68A	S103A	V104I	G159D	A232V	Q236R	Q245R	N248D	N252K				
N18S	V68A	S103A	V104I	G159D	A232V	Q236 Н	Q245R	N248D	N252K			
V68A	S103A	V104I	G159D	A232V	Ф236Н	Q245V	N248D	N252K				
V68A	N76D	S101T	S103A	V104I	G159D	T213R	N218S	A232V	Ф236Н	Q245R	T260A	
V68A	S103A	V104I	G159D	A228V	A232V	Q236 Н	Q245R	N248D	N252K			
T33S	V68A	N76D	S103A	V1041	G159D	A232V	Ф236Н	Q245R	N248D	N252K		
V68A	N76D	E89D	S103A	V104I	G159D	P210L	T213R	A232V	Ф236Н	Q245R	T260A	
G61E	V68A	N76D	S103A	V104I	G159D	A232V	Q236 Н	Q245R	N248D	N252K		
S103A	V104I	G159D	V205I	P210I	A232V	Q236Н	Q245R					
G61E	V68A	S103A	V104I	S130A	G159D	A232V	Q236H	Q245R	N248D	N252K		
G61E	V68A	S103A	V104I	A133S	Q137R	G159D	A232V	Q236H	Q245R	N248D	N252K	
G61E	S103A	V104I	A133V	G159D	A232V	Q236Н	Q245R	N248D	N252K			
V68A	S103A	V104I	G159D	A232V	Q236 Н	Q245R	N248G	N252K				
V68A	S103A	V104I	G159D	N218S	A232V	Ф236Н	Q245R	N248D	N252K			
G61E	V68A	S103A	V104I	G159D	S160V	A232V	Q236H	Q245R	N248D	N252K		
S3L	G61E	V68A	Q92N	S103A	V104I	A232V	Q236H	Q245R	N248D	N252K		

G61E	V68A	S103A	V104I	G159D	S167F	A232V	Ф236Н	Q245R	N248D	N252K		
3/65	S103A	V104I	G159D	A232V	Ф236Н	Q245R	N248D	N252K				
A98D	S103A	V104I	G159D	A232V	Ф236Н	Q245R	N248D	N252K				
366S	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K				
S101E	S103A	V104I	G159D	A232V	О236Н	Q245R	N248D	N252K				
S101G	S103A	V104I	G159D	A232V	Ф236Н	Q245R	N248D	N252K				
G102A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			-	
S103A	V104I	S106E	G159D	A232V	Q236Н	Q245R	N248D	N252K				
S103A	V104I	Q109E	G159D	A232V	Q236 Н	Q245R	N248D	N252K				
S103A	V104I	G159D	A232V	Ф236Н	Q245R	N248D	N252K	N261R				
S103A	V104I	Q109R	G159D	A232V	Q236 Н	Q245R	N248D	N252K				
N62D	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K				
S103A	V104I	G159D	N184D	A232V	Q236H	Q245R	N248D	N252K				
S103A	V104I	G159D	S166D	A232V	Q236Н	Q245R	N248D	N252K				
S103A	V104I	G159D	L217E	A232V	Q236H	Q245R	N248D	N252K				
G20R	N62D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K		
N62D	S103A	V104I	G159D	T213R	A232V	0236Н	Q245R	N248D	N252K			
S103A	V104I	G159D	Q206R	L217E	A232V	Q236H	Q245R	N248D	N252K			
N62D	S103A	V104I	G159D	Q206R	A232V	0236Н	Q245R	N248D	N252K			

S103A	V104I	S130G	G159D	A232V	Ф236Н	Q245R	N248D	N252K			
S103A	V104I	P131V	G159D	A232V	Q236Н	Q245R	N248D	N252K			
K27N	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
T38G	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
T38A	N76D	S103A	V104I	G159D	T213R	A232V	Q236 Н	Q245R	T260A		
V68A	U36D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	T260A	E271G	
V68A	N76D	S103A	V104I	G159D	Y209W	T213R	A232V	Ф236Н	Q245R	T260A	
V68A	N76D	S103A	V104I	G159D	P210I	T213R	A232V	Ф236Н	Q245R	T260A	
V68A	N76D	S103A	V104I	G159D	V205I	T213R	A232V	Ф236Н	Q245R	T260A	
V68A	N76D	S103A	V104I	G159D	P210I	A232V	Q236H	Q245R	T260A		
V68A	S103A	V104I	G159D	T213R	A232V	О236Н	Q245R	T260A			
N76D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	T260A			
V68A	S103A	V104I	G159D	Y209W	A232V	Ω236Н	Q245R				-
V68A	S103A	V104I	G159D	P210I	A232V	Ф236Н	Q245R				
V68A	S103A	V104I	G159D	A230V	A232V	Q236 Н	Q245R				
V68A	S103A	V104I	G159D	L126F	A232V	Q236 Н	Q245R				
V68A	S103A	V1041	G159D	V205I	A232V	Q236Н	Q245R			-	
V68A	S103A	V104I	G159D	P210L	A232V	Q236Н	Q245R				
S103A	V104I	G159D	A230V	0236Н	Q245R						

V68A S	S103A	V104I	G159D	A232V	Ф236Н	Q245R	T260A				
S103A \	V104I	G159D	A232V	Ф236Н	Q245R						
V68A S	S103A	V104I	G159D	A174V	A232V	Q236H	Q245R	L257V			
V68A S	S103A	V104I	G159D	A194S	A232V	Q236H	Q245R	L257V			
V68A S	S103A	V104I	G159D	Y209W	A232V	Q236H	Q245R	L257V			
S103A	V104I	G159D	A232V	Ф236Н	Q245R	L257V	-				
V68A	N76D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	T260A	N261W	-
V68A S	S103A	V104I	G159D	A232V	Ф236Н	Q245R	L257V	N261W			
S103A	V104I	G159D	T213R	A232V	Ф236Н	Q245R	T260A				
S103A	V104I	G159D	P210I	A232V	α236Н	Q245R	N248D	N252K			
S103A \	V104I	G159D	Y209W	A232V	Q236 Н	Q245R	L257V				
V68A	N76D	S103A	V104I	G159D	P210L	T213R	A232V	Q236H	Q245R	T260A	
Q12R S	S103A	V104I	G159D	Y209W	T213R	A232V	Q236H	Q245R	T260A		
S103A \	V104I	Y209W	A232V	Q236 Н	Q245R	L257V					
S103A \	V104I	G159D	V205I	P210I	T213R	A232V	Q236H	Q245R	T260A		
S103A \	V104I	G159D	V2051	Y209W	A232V	Q236 Н	Q245R	T260A			
V68A S	S103A	V104I	G159D	V205I	Y209W	P2101	A232V	Q236H	Q245R		
S103A \	V104I	G159D	V205I	Y209W	P2101	A232V	Q236H	Q245R	L257V		
S103A \	V104I	G159D	V2051	Y209W	A232V	Q236 Н	Q245R	L257V			

V68A	S103A	V104I	G159D	V205I	Y209W	P210I	A232V	Q236 Н	Q245R	T260A	
S103A	V104I	G159D	V2051	Y209W	P210I	A232V	Ф236Н	Q245R			
S103A	V104I	G159D	Y209W	P2101	A232V	α236Н	Q245R				
S103A	V104I	G159D	V205I	P210I	A232V	Ф236Н	Q245R				
V68A	S103A	V104I	S128L	G159D	A232V	α236Н	Q245R				
A48V	S103A	V104I	G159D	A230V	Q236H	Q245R					
A48V	V68A	S103A	V104I	G159D	Y209W	A232V	Ф236Н	Q245R			
A48V	V68A	S103A	V104I	G159D	A232V	Q236Н	Q245R	N248D	N252K		
A48V	V68A	S103A	V104I	G159D	A232V	Q236 Н	Q245R	L257V	N261W		
G102A	S103A	V104I	G159D	S212G	A232V	α236Н	Q245R	N248D	N252K		-
Q12R	G102A	S103A	V104I	G159D	S212G	A232V	Q236 Н	Q245R	N248D	N252K	
S101G	G102A	S103A	V104I	G159D	S212G	A232V	Q236H	Q245R	N248D	N252K	
A98L	G102A	S103A	V1041	G159D	S212G	A232V	Q236 Н	Q245R	N248D	N252K	
G102A	S103A	V104I	G159D	T213R	A232V	Ф236Н	Q245R	N248D	N252K		
S103A	V104I	P131V	G159D	A232V	Ф236Н	Q245R	N248D	N252K			
S103A	V104I	G159D	N184S	A232V	Q236 Н	Q245R	N248D	N252K			
S103A	V104I	G159D	N184G	A232V	Q236Н	Q245R	N248D	N252K			
S103A	V104I	G159D	A232V	Q236 Н	V244T	Q245R	N248D	N252K			
S103A	V104I	G159D	A232V	Q236Н	V244A	Q245R	N248D	N252K			

N62D	S103A	V104I	G159D	T213R	A232V	Ф236Н	Q245R	N248D	N252K	S256R		
Q12R	N62D	S103A	V104I	G159D	T213R	A232V	Q236H	Q245R	N248D	N252K		
S101G	S103A	V104I	G159D	N185D	A232V	Ф236Н	Q245R	N248D	N252K			
S101G	S103A	V104I	G159D	Q206E	A232V	Ф236Н	Q245R	N248D	N252K			
S101G	S103A	V104I	G159D	T213Q	A232V	О236Н	Q245R	N248D	N252K			
A98L	G102A	S103A	V104I	G159D	A232V	Q236H	Q245R	N248D	N252K			
S101G	G102A	S103A	V104I	G159D	A232V	Q236 Н	Q245R	N248D	N252K			
A98L	G102A	S103A	V104I	G159D	S212G	A232V	Ф236Н	Q245R	N248D	N252K		
A98L	G102A	S103A	V104I	G159D	S212G	A232V	Q236H	N248D	N252K			
N62D	S103A	V104I	Q109R	G159D	T213R	A232V	Ф236Н	Q245R	N248D	N252K		
N62D	S103A	V104I	G159D	S212G	T213R	A232V	Q236H	Q245R	N248D	N252K		
N62D	S101G	S103A	V104I	G159D	S212G	T213R	A232V	Ф236Н	Q245R	N248D	N252K	
S103A	V104I	G159D	A232V	Q245R	N248D	N252K						
S103A	V104I	G159D	A230V	Q245R								
N62D	S103A	V104I	S130G	G159D	T213R	A232V	Ф236Н	Q245R	N248D	N252K		
S101G	S103A	V104I	S130G	G159D	A232V	Q236 Н	Q245R	N248D	N252K			1
S101G	S103A	V104I	S128G	G159D	A232V	Ф236Н	Q245R	N248D	N252K			
S101G	S103A	V104I	S128L	G159D	A232V	Q236Н	Q245R	N248D	N252K			
N62D	S101G	S103A	V104I	G159D	T213R	A232V	а 236Н	Q245R	N248D	N252K		

N62D	S103A	V104I	S128G	G159D	T213R	A232V	Q236 Н	Q245R	N248D	N252K		
N62D	S103A	V104I	S128L	G159D	T213R	A232V	Ф236Н	Q245R	N248D	N252K		
S101G	S103A	V104I	G159D	A232V	Q236Н	Q245R	N248D	N252K	T260A			
S101G	S103A	V104I	P131V	G159D	A232V	Ф236Н	Q245R	N248D	N252K			
A98V	S101G	S103A	V104I	G159D	A232V	Ф236Н	Q245R	N248D	N252K			
S99G	S101G	S103A	V104I	G159D	A232V	α236Н	Q245R	N248D	N252K			
S101G	S103A	V104I	G159D	S212G	A232V	Ω236Н	Q245R	N248D	N252K			
S101G	S103A	V104I	G159D	Y209W	A232V	Ω236Н	Q245R	N248D	N252K			
S101G	S103A	V104I	G159D	P210I	A232V	Ф236Н	Q245R	N248D	N252K			
S101G	S103A	V104I	G159D	V205I	A232V	Ф236Н	Q245R	N248D	N252K			
S101G	S103A	V104I	G159D	A230V	Q236H	Q245R						
S101G	S103A	V104I	G159D	A194P	A232V	Q236 Н	Q245R	N248D	N252K			
N76D	S101G	S103A	V104I	G159D	A194P	A232V	Ф236Н	Q245R	N248D	N252K		
S101G	S103A	V104I	G159D	A230V	A232V	Ф236Н	Q245R	N248D	N252K			
N62D	S103A	V104I	G159D	N185D	Q206E	T213R	A232V	Q236H	Q245R	N248D	N252K E271Q	E271Q

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It is a further object to provide DNA sequences encoding such protease variants, as well as expression vectors containing such variant DNA sequences.

Still further, another object of the invention is to provide host cells transformed with such vectors, as well as host cells which are capable of expressing such DNA to produce protease variants either intracellularly or extracellularly.

There is further provided a cleaning composition comprising a protease variant of the present invention.

Additionally, there is provided an animal feed comprising a protease variant of the present invention.

Also provided is a composition for the treatment of a textile comprising a protease variant of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1 A-C depict the DNA and amino acid sequence for *Bacillus* amyloliquefaciens subtilisin and a partial restriction map of this gene.

Fig. 2 depicts the conserved amino acid residues among subtilisins from Bacillus amyloliquefaciens (BPN)' and Bacillus lentus (wild-type).

Figs. 3A and 3B depict the amino acid sequence of four subtilisins. The top line represents the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens* subtilisin (also sometimes referred to as subtilisin BPN'). The second line depicts the amino acid sequence of subtilisin from *Bacillus subtilis*. The third line depicts the amino acid sequence of subtilisin from *B. licheniformis*. The fourth line depicts the amino acid sequence of subtilisin from *Bacillus lentus* (also referred to as subtilisin 309 in PCT WO89/06276). The symbol * denotes the absence of specific amino acid residues as compared to subtilisin BPN'.

Detailed Description of the Invention

Proteases are carbonyl hydrolases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "protease" means a naturally-occurring protease or a recombinant protease. Naturally-occurring proteases include α -aminoacylpeptide hydrolase, peptidylamino acid hydrolase, acylamino hydrolase, serine carboxypeptidase, metallocarboxypeptidase, thiol proteinase, carboxylproteinase and metalloproteinase. Serine, metallo, thiol and acid proteases are included, as well as endo and exo-proteas s.

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The present invention includes protease enzymes which are non-naturally occurring carbonyl hydrolase variants (protease variants) having a diff rent proteolytic activity, stability, substrate specificity, pH profile and/or performance characteristic as compared to the precursor carbonyl hydrolase from which the amino acid sequence of the variant is derived. Specifically, such protease variants have an amino acid sequence not found in nature, which is derived by substitution of a plurality of amino acid residues of a precursor protease with different amino acids. The precursor protease may be a naturally-occurring protease or a recombinant protease.

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The protease variants useful herein encompass the substitution of any of the nineteen naturally occurring L-amino acids at the designated amino acid residue positions. Such substitutions can be made in any precursor subtilisin (procaryotic, eucaryotic, mammalian, etc.). Throughout this application reference is made to various amino acids by way of common one - and three-letter codes. Such codes are identified in Dale, M.W. (1989), Molecular Genetics of Bacteria, John Wiley & Sons, Ltd., Appendix B.

The protease variants useful herein are preferably derived from a *Bacillus* subtilisin. More preferably, the protease variants are derived from *Bacillus lentus* subtilisin and/or subtilisin 309.

Subtilisins are bacterial or fungal proteases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally-occurring subtilisin or a recombinant subtilisin. A series of naturally-occurring subtilisins is known to be produced and often secreted by various microbial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related proteases the relative order of these amino acids, reading from the amino to carboxy terminus, is aspartate-histidine-s rin. In the chymotrypsin related proteases, the relative order, however, is histidine-aspartate-s rine. Thus, subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related

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proteases. Examples include but are not limited to the subtilisins identified in Fig. 3 herein. Generally and for purposes of the present invention, numbering of the amino acids in proteases corresponds to the numbers assigned to the mature *Bacillus* amyloliquefaciens subtilisin sequence presented in Fig. 1.

"Recombinant subtilisin" or "recombinant protease" refer to a subtilisin or protease in which the DNA sequence encoding the subtilisin or protease is modified to produce a variant (or mutant) DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally-occurring amino acid sequence. Suitable methods to produce such modification, and which may be combined with those disclosed herein, include those disclosed in US Patent RE 34,606, US Patent 5,204,015 and US Patent 5,185,258, U.S. Patent 5,700,676, U.S. Patent 5,801,038, and U.S. Patent 5,763,257.

"Non-human subtilisins" and the DNA encoding them may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include gram negative organisms such as *E. coli* or *Pseudomonas* and gram positive bacteria such as *Micrococcus* or *Bacillus*. Examples of eucaryotic organisms from which subtilisin and their genes may be obtained include yeast such as *Saccharomyces cerevisiae*, fungi such as *Aspergillus* sp.

A "protease variant" has an amino acid sequence which is derived from the amino acid sequence of a "precursor protease". The precursor proteases include naturally-occurring proteases and recombinant proteases. The amino acid sequence of the protease variant is "derived" from the precursor protease amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor protease rather than manipulation of the precursor protease enzyme *per se*. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein, as well as methods known to those skilled in the art (see, for example, EP 0 328299, WO89/06279 and the US patents and applications already referenced herein).

Specific substitutions of amino acids at one or more residue positions corresponding to residue positions selected from the group consisting of 62, 212, 230, 232, 252 and 257 of *Bacillus amyloliquefaciens* subtilisin are identified h rein.

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Preferred variants are those having combinations of substitutions at residue positions corresponding to positions of *Bacillus amyloliquefaciens* subtilisin in Table 1.

More preferred variants are those having combinations of substitutions at residue positions corresponding to positions of *Bacillus amyloliquefaciens* subtilisin in Table 2.

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Further preferred variants are those having combinations of substitutions at residue positions corresponding to positions of *Bacillus amyloliquefaciens* subtilisin in Table 3.

able 3

	260											245			259	260
	245	252		252	252			245	257		245	236	245	245	245	245
252	236	248		245	245	245	257	236	245	257	236	232	236	236	236	236
245	232	245	245	236	236	236	245	232	236	245	232	214	232	232	232	232
236	213	236	236	232	232	232	236	210	232	236	211	159	215	159	159	159
232	159	232	232	129	159	159	232	159	224	232	159	1 04	159	102	<u>\$</u>	\$
159	104	159	159	140	\$	\$	159	401	159	159	\$	103	104	103	1 03	103
104	103	5	\$	5	103	103	5	103	<u>5</u>	104	103	92	103	92	92	87
103	92	103	103	103	89	89	103	9/	103	103	9/	89	9/	89	89	9/
89	89	89	89	68	43	43	68	89	89	9/	89	12	89	12	22	89

	T ·	Τ	Ţ	1	Τ_	T -	Ι —	Т		Т	Τ	<u> </u>	I	_	1	1	1
				<u> </u>													
			_						<u> </u>				_		_		-
	245		251	272	245				252		252	252	252	252	252	252	252
261	236		248	245	236	256	245	245	248		248	248	248	248	248	248	248
245	232	245	245	236	232	245	236	236	245	252	245	245	245	245	245	245	245
236	159	236	236	232	206	236	232	232	236	248	236	236	236	236	236	236	236
232	\$	232	232	159	183	232	206	159	232	245	232	232	232	232	232	232	232
159	103	192	159	1 05	159	159	159	2	212	236	508	159	159	509	210	212	213
104	9/	159	147	103	401	401	\$	103	159	232	159	109	4	159	159	159	159
103	89	104	104	9/	103	103	103	9/	<u>4</u>	159	104	104	103	104	<u>\$</u>	\$	\$
9/	48	103	103	68	9/	9/	92	68	103	104	103	103	68	103	103	103	103
89	12	9/	9/	12	89	88	89	27	89	103	89	89	20	89	89	89	88

	<u> </u>			<u> </u>					
								260	
								245	
	252	252	252	255	256	260	252	236	252
252	248	248	248	252	252	252	248	232	248
248	245	245	245	248	248	248	245	213	245
245	236	236	236	245	245	245	236	210	236
236	232	232	232	236	236	236	232	159	232
232	215	216	159	232	232	232	228	104	218
213	159	159	104	159	159	159	159	103	159
104	104	104	103	104	104	104	104	68	104
103	103	103	68	103	103	103	103	76	103
89	89	89	20	99	89	88	88	89	89

These amino acid position numbers refer to those assigned to the mature Bacillus amyloliquefaciens subtilisin sequence presented in Fig. 1. The invention, however, is not limited to the mutation of this particular subtilisin but extends to precursor proteases containing amino acid residues at positions which are "equivalent" to the particular identified residues in Bacillus amyloliquefaciens subtilisin. In a preferred embodiment of the present invention, the precursor protease is Bacillus lentus subtilisin and the substitutions are made at the equivalent amino acid residue positions in B. lentus corresponding to those listed above.

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A residue (amino acid) position of a precursor protease is equivalent to a residue of *Bacillus amyloliquefaciens* subtilisin if it is either homologous (i.e., corresponding in position in either primary or tertiary structure) or analogous to a specific residue or portion of that residue in *Bacillus amyloliquefaciens* subtilisin (i.e., having the same or similar functional capacity to combine, react, or interact chemically).

In order to establish homology to primary structure, the amino acid sequence of a precursor protease is directly compared to the Bacillus amyloliquefaciens subtilisin primary sequence and particularly to a set of residues known to be invariant in subtilisins for which sequence is known. For example, Fig. 2 herein shows the conserved residues as between B. amyloliquefaciens subtilisin and B. lentus subtilisin. After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of Bacillus amyloliquefaciens subtilisin are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained. Siezen et al. (1991) Protein Eng. 4(7):719-737 shows the alignment of a large number of serine proteases. Siezen et al. refer to the grouping as subtilases or subtilisin-like serine proteases.

For example, in Fig. 3, the amino acid sequence of subtilisin from Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus licheniformis (carlsbergensis) and Bacillus lentus are aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a

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number of conserved residues contained in each sequence. These conserved residues (as between BPN' and *B. lentus*) are identified in Fig. 2.

These conserved residues, thus, may be used to define the corresponding equivalent amino acid residues of *Bacillus amyloliquefaciens* subtilisin in other subtilisins such as subtilisin from *Bacillus lentus* (PCT Publication No. W089/06279 published July 13, 1989), the preferred protease precursor enzyme herein, or the subtilisin referred to as PB92 (EP 0 328 299), which is highly homologous to the preferred *Bacillus lentus* subtilisin. The amino acid sequences of certain of these subtilisins are aligned in Figs. 3A and 3B with the sequence of *Bacillus amyloliquefaciens* subtilisin to produce the maximum homology of conserved residues. As can be seen, there are a number of deletions in the sequence of *Bacillus lentus* as compared to *Bacillus amyloliquefaciens* subtilisin. Thus, for example, the equivalent amino acid for Val165 in *Bacillus amyloliquefaciens* subtilisin in the other subtilisins is isoleucine for *B. lentus* and *B. licheniformis*.

"Equivalent residues" may also be defined by determining homology at the level of tertiary structure for a precursor protease whose tertiary structure has been determined by x-ray crystallography. Equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the precursor protease and *Bacillus amyloliquefaciens* subtilisin (N on N, CA on CA, C on C and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the protease in question to the *Bacillus amyloliquefaciens* subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

$$R factor = \frac{\sum_{h} |Fo(h)| - |Fc(h)|}{\sum_{h} |Fo(h)|}$$

Equivalent residues which are functionally analogous to a specific residue of Bacillus amyloliquefaciens subtilisin are defined as those amino acids of the precursor protease which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate binding or catalysis in a manner

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defined and attributed to a specific residue of the *Bacillus amyloliquefaciens* subtilisin. Further, they are those residues of the precursor protease (for which a tertiary structure has been obtained by x-ray crystallography) which occupy an analogous position to the extent that, although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie with 0.13nm of the corresponding side chain atoms of *Bacillus amyloliquefaciens* subtilisin. The coordinates of the three dimensional structure of *Bacillus amyloliquefaciens* subtilisin are set forth in EPO Publication No. 0 251 446 (equivalent to US Patent 5,182,204, the disclosure of which is incorporated herein by reference) and can be used as outlined above to determine equivalent residues on the level of tertiary structure.

Some of the residues identified for substitution are conserved residues whereas others are not. In the case of residues which are not conserved, the substitution of one or more amino acids is limited to substitutions which produce a variant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such substitutions should not result in a naturally-occurring sequence. The protease variants of the present invention include the mature forms of protease variants, as well as the pro- and prepro-forms of such protease variants. The prepro-forms are the preferred construction since this facilitates the expression, secretion and maturation of the protease variants.

"Prosequence" refers to a sequence of amino acids bound to the N-terminal portion of the mature form of a protease which when removed results in the appearance of the "mature" form of the protease. Many proteolytic enzymes are found in nature as translational proenzyme products and, in the absence of post-translational processing, are expressed in this fashion. A preferred prosequence for producing protease variants is the putative prosequence of *Bacillus* amyloliquefaciens subtilisin, although other protease prosequences may be used.

A "signal sequence" or "presequence" refers to any sequence of amino acids bound to the N-terminal portion of a protease or to the N-terminal portion of a proprotease which may participate in the secretion of the mature or pro forms of the proteas. This definition of signal sequence is a functional one, meant to include all thos amino acid sequences encoded by the N-terminal portion of the proteas gen which participate in the effectuation of the secretion of proteas under native

conditions. The present invention utilizes such sequences to effect the secretion of the protease variants as defined herein. One possible signal sequence comprises the first seven amino acid residues of the signal sequence from Bacillus subtilis subtilisin fused to the remainder of the signal sequence of the subtilisin from Bacillus lentus (ATCC 21536).

A "prepro" form of a protease variant consists of the mature form of the protease having a prosequence operably linked to the amino terminus of the protease and a "pre" or "signal" sequence operably linked to the amino terminus of the prosequence.

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"Expression vector" refers to a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of said DNA in a suitable host. Such control sequences include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

The "host cells" used in the present invention generally are procaryotic or eucaryotic hosts which preferably have been manipulated by the methods disclosed in US Patent RE 34,606 to render them incapable of secreting enzymatically active endoprotease. A preferred host cell for expressing protease is the Bacillus strain BG2036 which is deficient in enzymatically active neutral protease and alkaline protease (subtilisin). The construction of strain BG2036 is described in detail in US Patent 5,264,366. Other host cells for expressing protease include Bacillus subtilis 1168 (also described in US Patent RE 34,606 and US Patent 5,264,366, the disclosure of which are incorporated herein by reference), as well as any suitable Bacillus strain such as B. licheniformis, B. lentus, etc.

Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of either

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replicating vectors encoding the protease variants or expressing the desired protease variant. In the case of vectors which encode the pre- or prepro-form of the protease variant, such variants, when expressed, are typically secreted from the host cell into the host cell medium.

"Operably linked," when describing the relationship between two DNA regions, simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

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The genes encoding the naturally-occurring precursor protease may be obtained in accord with the general methods known to those skilled in the art. The methods generally comprise synthesizing labeled probes having putative sequences encoding regions of the protease of interest, preparing genomic libraries from organisms expressing the protease, and screening the libraries for the gene of interest by hybridization to the probes. Positively hybridizing clones are then mapped and sequenced.

The cloned protease is then used to transform a host cell in order to express the protease. The protease gene is then ligated into a high copy number plasmid. This plasmid replicates in hosts in the sense that it contains the well-known elements necessary for plasmid replication: a promoter operably linked to the gene in question (which may be supplied as the gene's own homologous promoter if it is recognized, i.e., transcribed, by the host), a transcription termination and polyadenylation region (necessary for stability of the mRNA transcribed by the host from the protease gene in certain eucaryotic host cells) which is exogenous or is supplied by the endogenous terminator region of the protease gene and, desirably, a selection gene such as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in antibiotic-containing media. High copy number plasmids also contain an origin of replication for the host, thereby enabling large numb is of plasmids to be ginerated in the cytoplasm without chromosomal limitations. Howev in it is within the scope herein to integrate multiple copies of the protease gene into host genome. This is facilitated by procaryotic and

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eucaryotic organisms which are particularly susceptible to homologous recombination.

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The gene can be a natural *B. lentus* gene. Alternatively, a synthetic gene encoding a naturally-occurring or mutant precursor protease may be produced. In 'such an approach, the DNA and/or amino acid sequence of the precursor protease is determined. Multiple, overlapping synthetic single-stranded DNA fragments are thereafter synthesized, which upon hybridization and ligation produce a synthetic DNA encoding the precursor protease. An example of synthetic gene construction is set forth in Example 3 of US Patent 5,204,015, the disclosure of which is incorporated herein by reference.

Once the naturally-occurring or synthetic precursor protease gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor protease. Such modifications include the production of recombinant proteases as disclosed in US Patent RE 34,606 and EPO Publication No. 0 251 446 and the production of protease variants described herein.

The following cassette mutagenesis method may be used to facilitate the construction of the protease variants of the present invention, although other methods may be used. First, the naturally-occurring gene encoding the protease is obtained and sequenced in whole or in part. Then the sequence is scanned for a point at which it is desired to make a mutation (deletion, insertion or substitution) of one or more amino acids in the encoded enzyme. The sequences flanking this point are evaluated for the presence of restriction sites for replacing a short segment of the gene with an oligonucleotide pool which when expressed will encode various mutants. Such restriction sites are preferably unique sites within the protease gene so as to facilitate the replacement of the gene segment. However, any convenient restriction site which is not overly redundant in the protease gene may be used, provided the gene fragments generated by restriction digestion can be reassembled in proper sequence. If restriction sites are not present at locations within a convenient distance from the selected point (from 10 to 15 nucleotides), such sites are generated by substituting nucleotid s in the gene in such a fashion that neither the reading frame nor the amino acids encoded are changed in the final construction. Mutation of the gene in order to change its sequence to conform to the desired sequence is accomplished by M13 primer extension in accord with generally known

methods. The task of locating suitable flanking regions and evaluating the needed changes to arrive at two convenient restriction site sequences is made routine by the redundancy of the genetic code, a restriction enzyme map of the gene and the large number of different restriction enzymes. Note that if a convenient flanking restriction site is available, the above method need be used only in connection with the flanking region which does not contain a site.

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Once the naturally-occurring DNA or synthetic DNA is cloned, the restriction sites flanking the positions to be mutated are digested with the cognate restriction enzymes and a plurality of end termini-complementary oligonucleotide cassettes are ligated into the gene. The mutagenesis is simplified by this method because all of the oligonucleotides can be synthesized so as to have the same restriction sites, and no synthetic linkers are necessary to create the restriction sites.

As used herein, proteolytic activity is defined as the rate of hydrolysis of peptide bonds per milligram of active enzyme. Many well known procedures exist for measuring proteolytic activity (K. M. Kalisz, "Microbial Proteinases," <u>Advances in Biochemical Engineering/Biotechnology</u>, A. Fiechter ed., 1988). In addition to or as an alternative to modified proteolytic activity, the variant enzymes of the present invention may have other modified properties such as K_m, k_{cat}, k_{cat}/K_m ratio and/or modified substrate specificity and/or modified pH activity profile. These enzymes can be tailored for the particular substrate which is anticipated to be present, for example, in the preparation of peptides or for hydrolytic processes such as laundry uses.

In one aspect of the invention, the objective is to secure a variant protease having altered, preferably improved wash performance as compared to a precursor protease in at least one detergent formulation and or under at least one set of wash conditions.

There is a variety of wash conditions including varying detergent formulations, wash water volume, wash water temperature and length of wash time that a protease variant might be exposed to. For example, detergent formulations used in different areas have different concentrations of their relevant components present in the wash water. For example, a European detergent typically has about 4500-5000 ppm of detergent components in the wash water while a Japanese detergent typically has approximately 667 ppm of detergent components in the wash

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water. In North America, particularly the United States, a detergent typically has about 975 ppm of detergent components present in the wash water.

A low detergent concentration system includes detergents where less than about 800 ppm of detergent components are present in the wash water. Japanese detergents are typically considered low detergent concentration system as they have approximately 667 ppm of detergent components present in the wash water.

A medium detergent concentration includes detergents where between about 800 ppm and about 2000ppm of detergent components are present in the wash water. North American detergents are generally considered to be medium detergent concentration systems as they have approximately 975 ppm of detergent components present in the wash water. Brazil typically has approximately 1500 ppm of detergent components present in the wash water.

A high detergent concentration system includes detergents where greater than about 2000 ppm of detergent components are present in the wash water. European detergents are generally considered to be high detergent concentration systems as they have approximately 4500-5000 ppm of detergent components in the wash water.

Latin American detergents are generally high suds phosphate builder detergents and the range of detergents used in Latin America can fall in both the medium and high detergent concentrations as they range from 1500 ppm to 6000 ppm of detergent components in the wash water. As mentioned above, Brazil typically has approximately 1500 ppm of detergent components present in the wash water. However, other high suds phosphate builder detergent geographies, not limited to other Latin American countries, may have high detergent concentration systems up to about 6000 ppm of detergent components present in the wash water.

In light of the foregoing, it is evident that concentrations of detergent compositions in typical wash solutions throughout the world varies from less than about 800 ppm of detergent composition ("low detergent concentration geographies"), for example about 667 ppm in Japan, to between about 800 ppm to about 2000 ppm ("medium detergent concentration geographies"), for example about 975 ppm in U.S. and about 1500 ppm in Brazil, to greater than about 2000 ppm ("high detergent concentration geographies"), for example about 4500 ppm to about 5000 ppm in Europe and about 6000 ppm in high suds phosphate builder geographies.

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The concentrations of the typical wash solutions are determined empirically. For example, in the U.S., a typical washing machine holds a volume of about 64.4 L of wash solution. Accordingly, in order to obtain a concentration of about 975 ppm of detergent within the wash solution about 62.79 g of detergent composition must be added to the 64.4 L of wash solution. This amount is the typical amount measured into the wash water by the consumer using the measuring cup provided with the detergent.

As a further example, different geographies use different wash temperatures. The temperature of the wash water in Japan is typically less than that used in Europe.

Accordingly one aspect of the present invention includes a protease variant that shows improved wash performance in at least one set of wash conditions.

In another aspect of the invention, it has been determined that substitution of an amino acid at one or more residue positions corresponding to residue positions selected from the group consisting of 62, 212, 230, 232, 252 and 257 of *Bacillus amyloliquefaciens* subtilisin are important in improving the wash performance of the enzyme.

These substitutions are preferably made in *Bacillus lentus* (recombinant or native-type) subtilisin, although the substitutions may be made in any *Bacillus* protease.

Based on the screening results obtained with the variant proteases, the noted mutations in *Bacillus amyloliquefaciens* subtilisin are important to the proteolytic activity, performance and/or stability of these enzymes and the cleaning or wash performance of such variant enzymes.

Many of the protease variants of the invention are useful in formulating various detergent compositions or personal care formulations such as shampoos or lotions. A number of known compounds are suitable surfactants useful in compositions comprising the protease mutants of the invention. These include nonionic, anionic, cationic, or zwitterionic detergents, as disclosed in US 4,404,128 to Barry J. Anderson and US 4,261,868 to Jiri Flora, et al. A suitable detergent formulation is that described in Example 7 of US Patent 5,204,015 (previously incorporated by reference). The art is familiar with the different formulations which can be used as cleaning compositions. In addition to typical cleaning compositions, it is readily understood that the protease variants of the present invention may be

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used for any purpose that native or wild-type proteases are used. Thus, these variants can be used, for example, in bar or liquid soap applications, dishcare formulations, contact lens cleaning solutions or products, peptide hydrolysis, waste treatment, textile applications, as fusion-cleavage enzymes in protein production, etc. The variants of the present invention may comprise enhanced performance in a detergent composition (as compared to the precursor). As used herein, enhanced performance in a detergent is defined as increasing cleaning of certain enzyme sensitive stains such as grass or blood, as determined by usual evaluation after a standard wash cycle.

Proteases of the invention can be formulated into known powdered and liquid detergents having pH between 6.5 and 12.0 at levels of about 0.01 to about 5% (preferably 0.1% to 0.5%) by weight. These detergent cleaning compositions can also include other enzymes such as known proteases, amylases, cellulases, lipases or endoglycosidases, as well as builders and stabilizers.

The addition of proteases of the invention to conventional cleaning compositions does not create any special use limitation. In other words, any temperature and pH suitable for the detergent is also suitable for the present compositions as long as the pH is within the above range, and the temperature is below the described protease's denaturing temperature. In addition, proteases of the invention can be used in a cleaning composition without detergents, again either alone or in combination with builders and stabilizers.

The present invention also relates to cleaning compositions containing the protease variants of the invention. The cleaning compositions may additionally contain additives which are commonly used in cleaning compositions. These can be selected from, but not limited to, bleaches, surfactants, builders, enzymes and bleach catalysts. It would be readily apparent to one of ordinary skill in the art what additives are suitable for inclusion into the compositions. The list provided herein is by no means exhaustive and should be only taken as examples of suitable additives. It will also be readily apparent to one of ordinary skill in the art to only use those additives which are compatible with the enzymes and other components in the composition, for example, surfactant.

When present, the amount of additive present in the cleaning composition is from about 0.01% to about 99.9%, preferably about 1% to about 95%, more preferably about 1% to about 80%.

The variant proteases of the present invention can be included in animal feed such as part of animal feed additives as described in, for example. US 5,612,055; US 5,314,692; and US 5,147,642.

One aspect of the invention is a composition for the treatment of a textile that includes variant proteases of the present invention. The composition can be used to treat for example silk or wool as described in publications such as RD 216,034; EP 134,267; US 4,533,359; and EP 344,259.

The following is presented by way of example and is not to be construed as a limitation to the scope of the claims.

All publications and patents referenced herein are hereby incorporated by reference in their entirety.

Example 1

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A large number of protease variants were produced and purified using methods well known in the art. All mutations were made in *Bacillus lentus* GG36 subtilisin. The variants are shown in Table 4.

Table 4

		,					N218I	N248D		·					A174V
M222S	V104I	V104I	1107V	V104I	1246V	V104I	N183D	V104I	V104I	N261D	S160T	S216C	V104I	V104I	V104I
V104I	S103A	S103A	V104I	S103A	V104I	S103A	V104I	S103A	S103A	V104I	V104I	V104I	S103A	S103A	S103A
S103A	A98E	S78T	S103A	N76D	S103A	N77D	S103A	N76D	N76D	S103A	S103A	S103A	N76D	N76D	U77D
N76D	U36D	Q92N	09ZN	V4E	N76D	Q9/2N	N76D	A16T	A1E	N76D	N76D	M76D	H17Q	S37T	N76D

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K237Q									,		N185D	T274A			S240T	
V104I	V104I	N183D	V104I	V104I	V104I	N184D	N252D	S259C	K251T	V104I	V104I	K237E	S160L	A228V	V104I	A254T
S103A	S103A	V104I	S103A	S103A	S103A	V104	V104I	V104I	V104I	S103A	S103A	V104I	V104I	V104I	S103A	V104I
U36D	N76D	S103A	N76D	N76D	N76D	S103A	S103A	S103A	S103A	P86S	N76D	S103A	S103A	S103A	U36D	S103A
T38S	187	N76D	R19L	A13V	R19C	N76D	U36D	N76D	N76D	N76D	172V	N76D	N76D	N76D	P55S	Q9/N
	N76D S103A V104I	N76D S103A V104I N76D S103A V104I	N76D S103A V104I N76D S103A V104I S103A V104I N183D	N76D S103A V104I N76D S103A V104I S103A V104I N183D N76D S103A V104I	N76D S103A V104I N76D S103A V104I N76D S103A V104I N76D S103A V104I	N76D \$103A V104I N76D \$103A V104I N76D \$103A V104I N76D \$103A V104I	N76D S103A V104I N76D S103A V104I S103A V104I N183D N76D S103A V104I N76D S103A V104I N76D S103A V104I S103A V104I S103A V104I	N76D \$103A \$104I N76D \$103A \$104I \$103A \$104I \$104I N76D \$103A \$104I N76D \$103A \$104I N76D \$103A \$104I \$103A \$104I \$104I \$103A \$104I \$104I	N76D \$103A V104I N76D \$103A V104I \$103A V104I N183D N76D \$103A V104I N76D \$103A V104I N76D \$103A V104I \$103A V104I N184D \$103A V104I N252D \$103A V104I \$259C	N76D \$103A V104I N76D \$103A V104I \$103A V104I N183D N76D \$103A V104I N76D \$103A V104I N76D \$103A V104I \$103A V104I N184D \$103A V104I N252D \$103A V104I \$259C \$103A V104I \$259C	N76D \$103A V104I N76D \$103A V104I \$103A V104I N183D N76D \$103A V104I N76D \$103A V104I N76D \$103A V104I \$103A V104I N184D \$103A V104I N252D \$103A V104I \$259C \$103A V104I K251T \$103A V104I K251T \$103A V104I K251T	N76D \$103A V104I N76D \$103A V104I \$103A V104I N183D N76D \$103A V104I N76D \$103A V104I \$103A V104I N184D \$103A V104I N252D \$103A V104I \$259C \$103A V104I \$251T \$103A V104I \$251T \$103A V104I \$251T \$103A V104I \$251T \$103A V104I \$251T	N76D S103A V104I N76D S103A V104I S103A V104I N183D N76D S103A V104I N76D S103A V104I S103A V104I N184D S103A V104I N252D S103A V104I S259C S103A V104I K251T P86S S103A V104I N76D S103A V104I S103A V104I K251T	N76D S103A V104I N76D S103A V104I S103A V104I N183D N76D S103A V104I N76D S103A V104I S103A V104I N184D S103A V104I N252D S103A V104I S259C S103A V104I K251T P86S S103A V104I S103A V104I K251T S103A V104I K251T S103A V104I K237E S103A V104I S160L	N76D S103A V104I N76D S103A V104I S103A V104I N183D N76D S103A V104I N76D S103A V104I S103A V104I N252D S103A V104I S259C S103A V104I K251T P86S S103A V104I N76D S103A V104I S103A V104I K251T S103A V104I K237E S103A V104I S160L S103A V104I S160L S103A V104I S160L	N76D S103A V104I N76D S103A V104I S103A V104I N183D N76D S103A V104I N76D S103A V104I S103A V104I N252D S103A V104I S259C S103A V104I K251T P86S S103A V104I S103A V104I K251T P86S S103A V104I S103A V104I K237E S103A V104I S160L S103A V104I S160L S103A V104I S160L S103A V104I S160L S103A V104I S160L

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												K251R					
				V177A								Q236R	K237E			N204T	
N204T	N204D	V104I	G159D	V104I	V104I	A270V	N185D	V104I	L262M	V104I	V104I	S166G	V104I	S130L	Q109R	V104I	D181N
1104N	V104I	S103A	V104I	S103A	S103A	V104I	V104I	S103A	V104I	S103A	S103A	V104I	S103A	V104I	V104I	S103A	V104I
S103A	S103A	N76D	S103A	N76D	Q9/N	S103A	S103A	N76D	S103A	S78P	N76D	S103A	N76D	S103A	S103A	S99R	S103A
N76D	N76D	N43S	M76D	R10H	T58S	N76D	M76D	K27N	N76D	M76D	S24P	N76D	H17L	N76D	N76D	N76D	N76D

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												S265G				
					N183I		·		Y263H			H249Q	E271V			
E271V	N261Y			S242T	N116K				Q182R	A272S	1246V	Q206R	N238Y		11987	Q182R
S212P	N252K	S242T	E271Q	V104I	V104I	G258R	E271G	V104	V104I	Q182R	Q109R	V104I	Q137R		Q182R	V104I
V104I	V104I	V104I	V104I	S103A	S103A	V104I	V104I	S103A	S103A	V104I	V104	S103A	V104I	A228T	V104I	S103A
S103A	S103A	S103A	S103A	U36D	N76D	S103A	S103A	N76D	N76D	S103A	S103A	S87G	S103A	V104I	S103A	N76D
N76D	092N	M76D	N76D	Q12R	N43S	N76D	N76D	G61R	T38S	N76D	M76D	M76D	N76D	S103A	N76D	L21M
	S103A V104I S212P	S103A V104I S212P S103A V104I N252K	S103A V104I S212P S103A V104I N252K S103A V104I S242T	\$103A V104I \$212P \$103A V104I N252K \$103A V104I \$242T \$103A V104I E271Q	\$103A V104I \$212P \$103A V104I N252K \$103A V104I \$242T \$103A V104I E271Q N76D \$103A V104I	\$103A V104I \$212P E271V \$103A V104I N252K N261Y \$103A V104I \$242T \$242T \$103A V104I \$271Q \$242T N76D \$103A V104I \$242T N76D \$103A V104I \$242T	\$103A V104I \$212P E271V \$103A V104I N252K N261Y \$103A V104I \$242T \$242T \$103A V104I \$271Q \$242T \$103A V104I \$258R	\$103A V104I \$212P E271V \$103A V104I \$242T \$242T \$103A V104I \$242T \$242T N76D \$103A V104I \$242T N76D \$103A V104I \$242T N76D \$103A V104I \$242T \$103A V104I \$258R \$103A V104I \$258R	\$103A V104I \$212P E271V \$103A V104I \$242T \$242T \$103A V104I \$242T \$242T N76D \$103A V104I \$242T N76D \$103A V104I \$242T N76D \$103A V104I \$242T \$103A V104I \$258R \$103A N76D \$103A V104I \$271G \$103A V104I \$271G	\$103A V104I \$212P E271V \$103A V104I N252K N261Y \$103A V104I \$242T \$103A V104I \$242T \$103A V104I \$242T \$103A V104I \$242T \$103A V104I \$258R \$103A V104I \$271G \$103A V104I \$271G \$103A V104I \$271G \$103A V104I \$271G \$103A V104I \$271G	\$103A V104I \$212P E271V \$103A V104I N252K N261Y \$103A V104I \$242T \$103A V104I \$242T \$103A V104I \$242T \$103A V104I \$242T \$103A V104I \$258R \$103A V104I \$271G \$103A V104I \$271G \$103A V104I \$182R \$103A V104I \$182R	\$103A V104I \$212P E271V \$103A V104I \$242T N261Y \$103A V104I \$242T \$242T \$103A V104I \$242T \$103A V104I \$242T \$103A V104I \$242T \$103A V104I \$258R \$103A V104I \$258R \$103A V104I \$271G \$103A V104I \$272S \$103A V104I \$272S \$103A V104I \$272S \$103A V104I \$272S \$103A V104I \$246V	\$103A V104I \$212P E271V \$103A V104I N252K N261Y \$103A V104I \$242T \$103A V104I \$242T \$103A V104I \$242T \$103A V104I \$242T \$103A V104I \$258R \$103A V104I \$258R \$103A V104I \$271G \$103A V104I \$272S \$103A V104I \$272S \$103A V104I \$272S \$103A V104I \$246V \$103A V104I \$206R	\$103A V104I \$212P E271V \$103A V104I N252K N261Y \$103A V104I \$242T \$2503A \$103A V104I \$242T \$242T \$103A V104I \$242T \$242T \$103A V104I \$242T \$242T \$103A V104I \$242T \$242T \$103A V104I \$271G \$271G \$103A V104I \$271G \$271G \$103A V104I \$272S \$272S \$103A V104I \$272S \$272S \$103A V104I \$266F \$271V \$103A V104I \$206F \$271V \$103A V104I \$272S \$271V	\$103A V104I \$212P E271V \$103A V104I N252K N261Y \$103A V104I \$242T \$242T \$103A V104I \$242T \$242T \$103A V104I \$242T \$242T \$103A V104I \$242T \$250T \$103A V104I \$256R \$242T \$103A V104I \$242T \$272S \$103A V104I \$272S \$272S \$103A V104I \$246V \$272S \$103A V104I \$206R \$271V \$103I \$228T \$271V	\$103A V104I \$212P E271V \$103A V104I N252K N261Y \$103A V104I \$242T \$242T \$103A V104I \$242T \$183I \$103A V104I \$242T \$183I \$103A V104I \$242T \$183I \$103A V104I \$242T \$182I \$103A V104I \$242T \$182I \$103A V104I \$246V \$182I \$103A V104I \$246V \$182I \$103A V104I \$206R \$1246V \$103A V104I \$206R \$1249Q \$103A V104I \$137R \$1249Q \$103A V104I \$137R \$1249Q \$103A V104I \$137R \$1249Q \$103A V104I \$137R \$182R \$103A V104I \$188V \$188V

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										K251Q	N252D	K251T				
										L217E	L217E	N185D	V244A			
N248S	Q206R		G258R	E271G	N261D	Q206E	Q206E			G159D	G159D	A133T	Q206E	S188E	A158E	N185D
Q137R	V104I	Q206R	S212P	V104I	Q206E	V104I	V104I	A158E	Q206E	V104I	V104I	V104I	G159D	V104I	V104I	V104I
V104I	S103A	V104I	V104I	S103A	V104I	S103A	S103A	V104I	V104I	S103A	S103A	S103A	V104I	S103A	S103A	S103A
S103A	N76D	S103A	S103A	N76D	S103A	N76D	N77D	S103A	S103A	N76D	N76D	N77D	S103A	N76D	N76D	DZZN
N76D	A13T	N76D	N76D	T58S	M76D	V4E	M76D	N76D	M76D	V4E	V4E	U36D	U36D	V4E	V4E	M76D
	S103A V104I Q137R	S103A V104I Q137R N76D S103A V104I	S103A V104I Q137R N76D S103A V104I S103A V104I Q206R	S103A V104I Q137R N76D S103A V104I S103A V104I Q206R S103A V104I S212P	S103A V104I Q137R N76D S103A V104I S103A V104I Q206R S103A V104I S212P N76D S103A V104I	S103A V104I Q137R N76D S103A V104I S103A V104I G206R S103A V104I S212P N76D S103A V104I S103A V104I	S103A V104I Q137R N76D S103A V104I S103A V104I S212P N76D S103A V104I S103A V104I Q206E N76D S103A V104I	S103A V104I Q137R N76D S103A V104I S103A V104I S212P N76D S103A V104I S103A V104I Q206E N76D S103A V104I	S103A V104I Q137R N76D S103A V104I S103A V104I S212P N76D S103A V104I S103A V104I Q206E N77D S103A V104I S103A V104I A158E	S103A V104I Q137R N76D S103A V104I S103A V104I S212P N76D S103A V104I S103A V104I Q206E N77D S103A V104I S103A V104I A158E S103A V104I A158E	\$103A V104I Q137R N248S N76D \$103A V104I Q206R \$103A V104I Q206R C258R N76D \$103A V104I E271G \$103A V104I G206E N261D N76D \$103A V104I Q206E N77D \$103A V104I Q206E \$103A V104I Q206E \$103A V104I Q206E \$103A V104I G206E \$103A V104I G206E	\$103A V104i Q137R N248S N76D \$103A V104i Q206R \$103A V104i \$212P G258R \$103A V104i E271G \$103A V104i E271G \$103A V104i Q206E \$103A V104i G159D \$103A V104i G159D \$103A V104i G159D \$105B \$103A V104i \$105B \$103A V104i \$105B \$103A V104i	\$103A V104I Q137R N248S N76D \$103A V104I Q206R \$103A V104I \$212P G258R \$103A V104I E271G \$103A V104I E271G \$103A V104I Q206E \$103A V104I Q206E \$103A V104I Q206E \$103A V104I Q206E \$103A V104I G159D L217E \$103A V104I G159D L217E	\$103A V104I Q137R N248S N76D \$103A V104I Q206R \$103A V104I \$212P G258R \$103A V104I \$212P G258R \$103A V104I \$212P G258R \$103A V104I \$206E \$200E \$103A V104I \$206E \$200E \$103A V104I \$206E \$200E \$103A V104I \$206E \$200E \$103A V104I \$159D \$217E \$103A V104I \$159D \$217E \$103A V104I \$159D \$217E \$103A V104I \$133T \$185D \$103A V104I \$133T \$14A	\$103A V104I Q137R N248S N76D \$103A V104I Q206R \$103A V104I G206R C258R \$103A V104I E271G C206E \$103A V104I G206E C206E \$103A V104I Q206E C206E \$103A V104I Q206E C217E \$103A V104I G159D L217E \$103A V104I G159D L217E \$103A V104I G159D L217E \$103A V104I G159D L217E \$103A V104I G159D Q206E \$103A V104I G159D Q206E \$103A V104I G159D Q204A \$103A V104I G159D Q204A	\$103A V104I Q137R N248S N76D \$103A V104I Q206R \$103A V104I \$212P G258R \$103A V104I \$212P G258R \$103A V104I \$212P G258R \$103A V104I \$206E \$206E \$103A V104I \$206E \$206E \$103A V104I \$159D \$217E \$103A V104I \$159D \$244A \$103A V104I \$188E \$244A \$105D \$103A V104I \$18E

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													E271V	E271V			
	G159D	Q236H		G159D					E271V		E271V	E271V	S212P	N243S			
K251T	L111M	G159D	G159D	V104I	G159D	G159D	N238S	T224A	V268F		S212P	Q245L	S141N	Q236L	Q245R	P210L	V104I
Q206E	V104I	V104I	V104I	S103A	V104I	G146S	G159D	G159D	S212P	V104I	V104I	S212P	T134S	S212P	Q109R	Q109R	S103A
V104I	S103A	S103A	S103A	N76D	S103A	V104I	V104	V104I	V104I	S103A	S103A	V104I	V104I	V104I	V104I	V104I	N76D
S103A	Q92N	U36N	N76D	N62H	M76D	S103A	S103A	S103A	S103A	E89A	S87R	S103A	S103A	S103A	S103A	S103A	N62S
N76D	A48T	V68A	L42V	Q12H	L42I	N76D	N76D	N76D	M76D	N76D	M76D	N76D	N76D	M76D	U36N	N76D	G20V

								Q245R									
			E271V					Q236H		T253K	Q236H				H249Y		
	E271V	Q245R	Q236H				Q236H	G159D	Q236H	Q236Н	N184S	N243I	Q245L		Q236H	H249Q	
	Q236H	Q236H	L217I			Q236R	G159D	V1211	G159D	Y209S	G159D	Q236H	Q236H	G159D	G159D	Q236H	
Ф236Н	G159D	G159D	G159D	V104I		G159D	V104I	A114V	V104I	G159D	N117K	G159D	G159D	A142V	N123S	G159D	Q245R
V104I	V104I	V104I	V104I	S103A	V104I	V104I	S103A	S103A	S103A	V104I	V104I	V104I	V104I	V104I	V104I	V104I	M222S
S103A	S103A	S103A	S103A	N76D	S103A	S103A	N76D	N76D	D92N	S103A	S103A	S103A	S103A	S103A	S103A	S103A	V104I
N76D	N76D	N76D	N76D	V68A	N76D	N76D	L75R	N76D	V68A	N76D	U36D	N76D	N76D	N76D	N76D	N76D	S103A
V68A	V68A	V68A	V68A	H170	V68A	V68A	V68A	V68A	Q12R	V68A	V68A	V68A	V68A	V68A	V68A	V68A	U36D

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											T255S		Q245R		Q245R		Q245R
										N261D	Q245R	R247H	Q236H	Q245R	Q236 Н	Q245R	Q236H
			Y263F							Q245R	Ф236Н	Q245R	N204D	Q236 Н	N218D	Ф236Н	V203A
H249R			K237R		E271D			N248S		Ф236Н	G159D	Ф236Н	A174V	N204D	G159D	A232V	A1941
M222S	M222S	Y263F	M222S	M222S	M222S	M222S	M222S	M222S	H249R	G159D	S141N	G159D	G159D	G159D	A133V	G159D	G159D
Q12R	N173R	M222S	V104I	Q109R	Q109R	V104I	Q137R	Q109R	M222S	V104I	V104I	V104I	V104I	V104I	V104	V104	V104I
V104I	V104I	V104I	S103A	V104I	V104I	S103A	V104I	V104I	V104I	S103A	S103A	S103A	S103A	S103A	S103A	S103A	S103A
S103A	S103A	S103A	N76D	S103A	S103A	N76D	S103A	S103A	S103A	N76D	N76D	N76D	N76D	N76D	N76D	N76D	U36D
N76D	N76D	N76D	L21M	N76D	N76D	G61R	N76D	N76D	N76D	V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A

				T260A												R275S	
		Q245R		Q245R						N252K		N252K	N252K		N252K	N252K	L262M
		Q236 Н	N252K	Q236H						N248D		Q245R	Q245R	Q245R	Q245R	Q245R	N248S
		A232V	Q245R	A232V	Q245R		Q245R			Q245R	Q245R	Ф236Н	Q236H	Q236H	Q236Н	Q236H	Q245R
Q245R		G159D	Ф236Н	T213R	V244I	Q245R	M222S			Q236H	Q236H	A232V	A232V	A232V	A232V	A232V	M222S
M222S	Q245R	V104I	A232V	G159D	M222S	P210T	S130T	V104I		A232V	A232V	G159D	G159D	G159D	G159D	G159D	S130T
V104I	A232V	S103A	G159D	V104I	1104T	M222S	1104T	S103A	N184D	G159D	G159D	N140D	V104I	V104I	V104I	V104I	1104T
S103A	V104I	N76D	V104I	S103A	S103A	S103A	S103A	N76D	S103A	V104I	V104I	V104I	S103A	S103A	S103A	S103A	S103A
N76D	S103A	V68A	S103A	N76D	N76D	N76D	N76D	V68A	N76D	S103A	S103A	S103A	V68A	V68A	V68A	S87G	N76D
Q12R	N76D	S24T	V68A	V68A	Q12R	Q12R	Q12R	T22K	V68A	V68A	V68A	V68A	N43S	N43K	N43D	V68A	Q12R

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					N269D		Q245R							-			
	L262S				L262S	K251Q	N243D									Q245R	
Q245R	Q245R	Q245R	N261D		Q245R	Q245R	M222S	V268A	Q245R	L257V	Q245R	N248D	Q245R	Q245R	Q245R	Q236H	Q245R
M222S	V227A	M222S	Q245R		M222S	M222S	N185D	Q245R	P210S	Q245R	Q236H	Q245R	Q236H	Q236Н	K237E	A232V	Q236Н
A215V	M222S	A215T	M222S	Q245R	N218D	S130T	R170S	M222S	M222S	Q236H	A232V	Q236Н	A232V	A232V	Q236Н	G159D	A232V
S130T	S130T	S130T	S130T	M222S	S130T	1104T	S130T	S130T	S130T	A232V	G159D	A232V	G159D	V203E	A232V	V104I	N183D
1104T	1104T	1104T	1104T	S130T	1104T	S103A	1104T	1104T	1104T	G159D	N116D	G159D	V104I	G159D	G159D	S103A	G159D
S103A	S103A	S103A	S103A	1104T	S103A	N76D	S103A	S103A	S103A	V104I	V104I	V104I	S103A	V104I	V104I	N621	V104I
N76D	N76D	N76D	N76D	S103A	N76D	S57P	N76D	N76D	N76D	S103A	S103A	S103A	V68A	S103A	S103A	N76D	S103A
Q12R	Q12R	Q12R	Q12R	09ZN	Q12R	Q12R	Q12R	Q12R	Q12R	V68A	V68A	V68A	R10C	V68A	V68A	V68A	V68A

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																	Q245R
Q245R							Q245R	L257V		R275H		L257V		Q245R	Q245R	Q245R	Q 236H
Q236H	Q245R	Q245R	Q245R	Q245R	N248S	Q245R	Q236 Н	Q245R		L257V		Q245R	L257V	Q236H	Q236Н	Q236H	A232V
A232V	Q236H	Ф236Н	Q236H	Q236Н	Q245R	Q236H	A232V	Q236H	L257V	Q245R		Q236H	Q245R	A232V	A232V	A232V	Y214L
Q206L	A232V	A232V	A232V	A232V	Q236H	A232V	P210R	A232V	Q245R	Ф236Н		A232V	Ф236Н	Y209W	G211R	G211V	G159D
A174V	S188C	A230T	G159D	A215T	A232V	G159D	G159D	G159D	О236Н	A232V	R275H	T224A	A232V	G159D	G159D	G159D	V104I
G159D	G159D	G159D	V104I	G159D	G159D	V104I	V104I	V104I	A232V	G159D	L257V	G159D	G159D	V104I	V104I	V104I	S103A
V104I	V104I	V104I	S103A	V104I	V104I	S103A	S103A	S103A	V104I	V104I	V104I	V104I	V104I	S103A	S103A	S103A	N76D
S103A	S103A	S103A	A98T	S103A	S103A	N76D	M76D	N76D	S103A	S103A	S103A	S103A	S103A	N76D	N76D	N76D	V68A
V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A	M76D	V68A	N76D	V68A	N76D	V68A	V68A	V68A	Q12R

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		S259G	T260V					Q245R			K251R	A272S	Q245R				Q236H
Q245R	Q245R	Q245R	Q245R	N261G	N261W		Q245R	Q236H			N248S	Q245R	Q236H	S256R	Q245R	Q245R	A232V
Q236H	Q236H	Q236H	Q236H	Q245R	Q245R		Q236H	A232V		Q245R	Q245R	Q236H	A232V	Q245R	Q236H	Q236H	N185S
A232V	A232V	A232V	A232V	Q236 Н	Q236H	Q245R	A232V	G159D		Q236H	Q236H	A232V	Q206L	Q236H	A232V	A232V	R170S
A215R	G159D	G159D	G159D	A232V	A232V	S242P	P210L	V104I	Q245R	A232V	A232V	G159D	N183K	A232V	Q206R	G159D	G159D
G159D	V104I	V104I	V104I	G159D	G159D	Q236H	G159D	S103A	Q236Н	Y192F	G159D	V104I	G159D	G159D	G159D	V104I	N116T
V104I	S103A	S103A	S103A	V104I	V104I	A232V	V104I	N76D	A232V	G159D	V147I	S103A	V104I	V104I	V104I	S103A	V104I
S103A	N76D	N76D	N76D	S103A	S103A	14017	S103A	V68A	V104	V104	V104I	N76D	S103A	S103A	S103A	U36D	S103A
N76D	V68A	V68A	S87R	N76D	N76D	S103A	N76D	A48V	S103A	S103A	S103A	V68A	N76D	M76D	N76D	V68A	N76D
V68A	Q12R	G20R	V68A	V68A	V68A	N76D	V68A	Q12R	N76D	M76D	N76D	Q12R	V68A	V68A	V68A	K27R	V68A

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			N252K											N252K			
N252K	N252K	N252K	N248D		N252K	N252K	N252K	N252K	NZ61D	N252K	N252K	N252K	N252K	N248D	N252K	N252K	N252K
N248D	N248D	N248D	Q245R		N248D	N248D	N248D	N248D	N252K	N248D	N248D	N248D	N248D	Q245R	N248D	N248D	N248D
Q245R	Q245R	Q245R	Q236H	N252K	Q245R	Q245R	Q245R	Q245R	N248D	Q245R	Q245R	Q245R	Q245R	Q236H	Q245R	Q245R	Q245R
Q236H	Q236H	Q236H	A232V	N248D	Ф236Н	Q236H	Q236H	Q236H	Q245R	Ф236Н	Q236H	Q236H	Q236H	A232V	Q236H	Q236H	Q236H
A232V	A232V	A232V	N184D	Q245R	A232V	A232V	A232V	A232V	Ф236Н	A232V	A232V	A232V	A232V	P210L	A232V	A232V	A232V
G159D	G159D	S212P	G159D	Q236H	Y209W	G159D	G159D	Y209F	A232V	N185D	P210R	P210T	P210S	N185D	P210L	S212A	\$212G
V104I	V104I	G159D	N66S	A232V	G159D	Q109R	V104I	G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D
S103A	S103A	V104I	V104I	G159D	V104I	V104I	S103A	V104I	V104I	V104I	V104I	V104I	V104I	V104I	V104I	V104I	V104I
V68A	V68A	S103A	S103A	V104I	S103A	S103A	V68A	S103A	S103A	S103A	S103A	S103A	S103A	S103A	S103A	S103A	S103A
G61E	N43D	V68A	V68A	S103A	V68A	V68A	G20R	V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A

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N252K	N252K		N252K	N252K	N252K	N252K	N252K	N252K	N252K	N252K	N252K	N252K	N252K	N252K			N252F
N248D	N248D	N252K	N248D	N248D	N248D	N248D	N248D	N248D	N248D	N248D	N248D	N248D	K251V	N248D	N252F	N252L	N248D
Q245R	Q245R	N248D	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	N248D	Q245R	N248D	N248D	Q245R
Q236 Н	Q236H	Q245R	Q236H	Q236H	Q236H	Ф236Н	Q236H	Q236H	Q236H	Q236H	Q236H	Ф236Н	Q245R	Q236H	Q245R	Q245R	Q236H
A232V	A232V	Q236H	A232V	A232V	A232V	A232V	A232V	A232V	A232V	A232V	A232V	A232V	Q236Н	A232V	Q236H	Q236H	A232V
S212E	T213E	A232V	T213E	T213R	T213G	A215V	A215R	S216T	S216V	S216C	G159D	N173D	A232V	Q206R	A232V	A232V	G159D
G159D	G159D	T213S	G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	V104I	G159D	G159D	G159D	G159D	G159D	V104I
V104I	V104I	V104I	V104I	V104I	V104I	V104I	V104I	V104I	V104I	V104I	S103A	V104I	V104I	V104I	V104I	V104I	S103A
S103A	S103A	S103A	A103V	S103A	S103A	S103A	S103A	S103A	S103A	S103A	V68A	S103A	S103A	S103A	S103A	S103A	V68A
V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A	G20A	V68A	V68A	V68A	V68A	V68A	P55S

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																	T260A
							N269D	T260E									Q245R
T255V	S256N	S256E	S256R	T260R	L257R	G258D	N252K	N252K	N261R	N261D	N252K				N252K		Q236 Н
N252K	N252K	N252K	N252K	N252K	N252K	N252K	N248D	N248D	N252K	N252K	N248D			N252K	N248D	N252K	A232V
N248D	N248D	N248D	N248D	N248D	N248D	N248D	Q245R	Q245R	N248D	N248D	Q245R	N252K	N252K	N248D	Q245R	N248D	N218S
Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	Q236H	Q236H	Q245R	Q245R	Q236H	N248D	N248D	Q245R	Q236Н	Q245V	T213R
Q236 Н	Q236H	О236Н	Q236H	О236Н	Q236Н	Ф236Н	A232V	A232V	О236Н	Q236H	A232V	Q245R	Q245R	Q236R	A232V	Q236H	G159D
A232V	A232V	A232V	A232V	A232V	A232V	A232V	G159D	G159D	A232V	A232V	G159D	Q236Н	Q236H	A232V	G159D	A232V	V104I
G159D	G159D	G159D	G159D	G159D	G159D	G159D	V104I	N116S	G159D	G159D	V104I	A232V	A232S	G159D	V104I	G159D	S103A
V104I	V104I	V104I	V104I	V104I	V104I	V104I	S103A	V104I	V104I	V104I	S103A	V104I	G159D	V104I	S103A	V104I	S101T
S103A	S103A	S103A	S103A	S103A	S103A	S103A	V68A	S103A	S103A	S103A	N76D	S103A	V104I	S103A	V68A	S103A	N76D
V68A	V68A	V68A	V68A	V68A	V68A	V68A	187	V68A	V68A	V68A	V68A	V68A	S103A	V68A	N18S	V68A	V68A

		T260A				N252K											
	N252K	Q245R	N252K		N252K	N248D				N252K	N252K	N252K					
N252K	N248D	Q236H	N248D		N248D	Q245R	N252K		N252K	N248D	N248D	N248D					
N248D	Q245R	A232V	Q245R		Q245R	Q236Н	N248D	N252K	N248D	Q245R	Q245R	Q245R	N252K	N252K	N252K	N252K	N252K
Q245R	Q236H	T213R	Q236H	Q245R	Q236H	A232V	Q245R	N248G	Q245R	Q236H	Q236H	Q236H	N248D	N248D	N248D	N248D	N248D
Ф236Н	A232V	P210L	A232V	Q236Н	A232V	G159D	Q236Н	Q245R	Q236H	A232V	A232V	A232V	Q245R	Q245R	Q245R	Q245R	Q245R
A232V	G159D	G159D	G159D	A232V	G159D	Q137R	A232V	Ф236Н	A232V	S160V	V104I	S167F	Q236H	Q236H	Q236 Н	Q236 Н	Q236H
A228V	V104I	V104I	V104I	P210I	S130A	A133S	G159D	A232V	N218S	G159D	S103A	G159D	A232V	A232V	A232V	A232V	A232V
G159D	S103A	S103A	S103A	V205I	V104I	V104I	A133V	G159D	G159D	V104I	N76D	V104I	G159D	G159D	G159D	G159D	G159D
V104I	N76D	E89D	U36D	G159D	S103A	S103A	V104I	V104I	V104I	S103A	V68A	S103A	V104I	V104I	V104I	V104I	V104I
S103A	V68A	N76D	V68A	V104I	V68A	V68A	S103A	S103A	S103A	V68A	G61E	V68A	S103A	S103A	S103A	S103A	S103A
V68A	T33S	V68A	G61E	S103A	G61E	G61E	G61E	V68A	V68A	G61E	S3L	G61E	G97E	A98D	S99E	S101E	S101G

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									N252K								
									N248D	N252K	N252K	N252K					T260A
N252K	N252K	N252K	N261R	N252K	N252K	N252K	N252K	N252K	Q245R	N248D	N248D	N248D	N252K	N252K	N252K	N252K	Q245R
N248D	N248D	N248D	N252K	N248D	N248D	N248D	N248D	N248D	Q236H	Q245R	Q245R	Q245R	N248D	N248D	N248D	N248D	Q236H
Q245R	Q245R	Q245R	N248D	Q245R	Q245R	Q245R	Q245R	Q245R	A232V	Q236H	Q236H	Q236H	Q245R	Q245R	Q245R	Q245R	A232V
Q236 Н	Q236H	Q236 Н	Q245R	Q236H	Q236H	Q236H	Q236H	Q236H	T213R	A232V	A232V	A232V	Q236H	Q236H	Q236H	Q236H	T213R
A232V	A232V	A232V	Q236H	A232V	A232V	A232V	A232V	A232V	G159D	T213R	L217E	Q206R	A232V	A232V	A232V	A232V	G159D
G159D	G159D	G159D	A232V	G159D	G159D	N184D	S166D	L217E	V104I	G159D	Q206R	G159D	G159D	G159D	G159D	G159D	V104I
V104I	S106E	Q109E	G159D	Q109R	V104I	G159D	G159D	G159D	S103A	V104I	G159D	V104I	S130G	P131V	V104I	V104I	S103A
S103A	V104I	V104I	V104I	V104I	S103A	V104I	V104I	V104I	N62D	S103A	V104I	S103A	V104I	V104I	S103A	S103A	Q9/N
G102A	S103A	S103A	S103A	S103A	N62D	S103A	S103A	S103A	G20R	N62D	S103A	N62D	S103A	S103A	K27N	T38G	T38A

V104I G159D T213R A232V Q236H V104I G159D Y209W T213R A232V V104I G159D Y209W T213R A232V V104I G159D P210I T213R A232V V104I G159D P210I A232V Q236H G159D T213R A232V Q236H Q245R G159D T213R A232V Q236H Q245R G159D Y209W A232V Q236H Q245R G159D Y209W A232V Q236H Q245R G159D L126F A232V Q236H Q245R G159D L126F A232V Q236H Q245R G159D V206I A232V Q236H Q245R G159D V206I A232V Q236H Q245R G159D A232V Q236H Q245R G159D A232V Q236H Q245R A232V Q236H Q245R			, . -				-		,					,				
V104I G159D T213R A232V Q236H Q245R T260A V104I G159D Y209W T213R A232V Q236H Q245R V104I G159D Y209I T213R A232V Q236H Q245R V104I G159D V205I T213R A232V Q236H Q245R T260A G159D T213R A232V Q236H Q245R T260A C150A G159D T213R A232V Q236H Q245R T260A C150A G159D T213R A232V Q236H Q245R T260A C150A G159D T213R A232V Q236H Q245R C150A C150A G159D L126F A232V Q236H Q245R C150A C150A G159D V206I A232V Q236H Q245R C150A C150A A230V Q236H Q245R T260A C150A C150A C150A A232V <					: : :													
V104I G159D T213R A232V Q236H Q245R T260A V104I G159D Y209W T213R A232V Q236H Q245R V104I G159D Y209I T213R A232V Q236H Q245R V104I G159D V205I T213R A232V Q236H Q245R T260A G159D T213R A232V Q236H Q245R T260A C150A G159D T213R A232V Q236H Q245R T260A C150A G159D T213R A232V Q236H Q245R T260A C150A G159D T213R A232V Q236H Q245R C150A C150A G159D L126F A232V Q236H Q245R C150A C150A G159D V206I A232V Q236H Q245R C150A C150A A230V Q236H Q245R T260A C150A C150A C150A A232V <																		
V104I G159D T213R A232V Q236H Q245R V104I G159D Y209W T213R A232V Q236H V104I G159D P210I T213R A232V Q236H V104I G159D P210I A232V Q236H Q245R G159D T213R A232V Q236H Q245R T260A G159D T213R A232V Q236H Q245R T260A G159D T213R A232V Q236H Q245R T260A G159D P210I A232V Q236H Q245R T260A G159D P210I A232V Q236H Q245R T260A G159D V205I A232V Q236H Q245R T260A G159D V206I A232V Q236H Q245R T260A A232V Q236H Q245R T260A A232V Q236H G159D A232V Q236H Q245R T260A A232V	E271G	T260A	T260A	T260A														
V104I G159D T213R A232V V104I G159D Y209W T213R V104I G159D P210I T213R V104I G159D P210I A232V G159D T213R A232V Q236H G159D T213R A232V Q236H G159D T213R A232V Q236H G159D Y209W A232V Q236H G159D P210I A232V Q236H G159D L126F A232V Q236H G159D L205I A232V Q236H G159D V205I A232V Q236H G159D V205I A232V Q236H G159D V205I A232V Q236H G159D A232V<	T260A	Q245R	Q245R	Q245R	T260A													
V104I G159D T213R A232V V104I G159D Y209W T213R V104I G159D P210I T213R V104I G159D P210I A232V G159D T213R A232V Q236H G159D T213R A232V Q236H G159D T213R A232V Q236H G159D Y209W A232V Q236H G159D P210I A232V Q236H G159D L126F A232V Q236H G159D L205I A232V Q236H G159D V205I A232V Q236H G159D V205I A232V Q236H G159D V205I A232V Q236H G159D A232V<	Q245R	Q236H	Ф236Н	Q236Н	Q245R	T260A	T260A										L257V	L257V
V104I G159D T213R V104I G159D Y209W V104I G159D P210I V104I G159D P210I G159D T213R A232V G159D T213R A232V G159D T213R A232V G159D Y209W A232V G159D P210I A232V G159D L126F A232V G159D L230V A232V G159D V205I A232V G159D V205I A232V G159D V205I A232V G159D V205I A232V G159D A232V Q236H	Q236H	A232V	A232V	A232V	Q236H	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R		T260A		Q245R	Q245R
V104I G159D V104I G159D V104I G159D V104I G159D V104I G159D G159D T213R G159D T213R G159D Y209W G159D Y209W G159D Y209I G159D Y205I	A232V	T213R	T213R	T213R	A232V	Q236H	Q236H	Ф236Н	Q236Н	Q236H	Q236H	Ф236Н	Q236H		Q245R		Q236H	Q236Н
V104I V104I V104I V104I C159D G159D G159D G159D G159D G159D G159D G159D G159D G159D G159D G159D G159D G159D G159D G159D G159D G159D	T213R	Y209W	P210I	V205I	P210I	A232V	A232V	A232V	A232V	A232V	A232V	A232V	A232V	Q245R		Q245R	A232V	A232V
 	G159D	G159D	G159D	G159D	G159D	T213R	T213R	Y209W	P210I	A230V	L126F	V205I	P210L	Q236H	A232V	Q236Н	A174V	A194S
\$103A \$103A	V104I	V104I	V104I	V104I	V104I	G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	A230V	G159D	A232V	G159D	G159D
	S103A	S103A	S103A	S103A	S103A	V104I	V104I	V104I	V104I	V104I	V104I	V104I	V104I	G159D	V104I	G159D	V104I	V104
N76D N76D N76D S103A S103A S103A S103A S103A S103A S103A S103A S103A S103A S103A	N76D	Q9/N	Q92N	092N	N76D	S103A	S103A	S103A	S103A	S103A	S103A	S103A	S103A	V104I	S103A	V104I	S103A	S103A
V68A V68A V68A V68A V68A V68A V68A V68A V68A V68A V68A V68A V68A V68A V68A V68A V68A V68A V68A	V68A	V68A	V68A	V68A	V68A	V68A	N76D	V68A	V68A	V68A	V68A	V68A	V68A	S103A	V68A	S103A	V68A	V68A

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		N261W					T260A								T260A		
		T260A					Q245R	T260A		T260A		Q245R	L257V		Q245R		
L257V		Q245R	N261W		N252K		Q236H	Q245R		Q245R	T260A	Q236H	Q245R	L257V	Q236 Н	Q245R	
Q245R		Ф236Н	L257V	T260A	N248D	L257V	A232V	Q236 Н		Q236H	Q245R	A232V	Ф236Н	Q245R	A232V	Q236H	Q245R
Q236 Н	L257V	A232V	Q245R	Q245R	Q245R	Q245R	T213R	A232V	L257V	A232V	Q236H	P210I	A232V	Ф236Н	P2101	A232V	Q236H
A232V	Q245R	T213R	Q236H	Q236H	Q236H	Q236Н	P210L	T213R	Q245R	T213R	A232V	Y209W	P2101	A232V	Y209W	P2101	A232V
Y209W	Q236H	G159D	A232V	A232V	A232V	A232V	G159D	Y209W	Q236Н	P2101	Y209W	V205I	Y209W	Y209W	V205I	Y209W	P2101
G159D	A232V	V1041	G159D	T213R	P2101	Y209W	V104I	G159D	A232V	V205I	V205I	G159D	V205I	V205I	G159D	V205I	Y209W
V104I	G159D	S103A	V104I	G159D	G159D	G159D	S103A	V104I	Y209W	G159D	G159D	V104I	G159D	G159D	V104I	G159D	G159D
S103A	V104	N76D	S103A	V104I	V104I	V104I	N76D	S103A	V104I	V104I	V104I	S103A	V104I	V104I	S103A	V104I	V104I
V68A	S103A	V68A	V68A	S103A	S103A	S103A	V68A	Q12R	S103A	S103A	S103A	V68A	S103A	S103A	V68A	S103A	S103A

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							N252K	N252K	N252K							S256R	N252K
				N252K	N261W	N252K	N248D	N248D	N248D	N252K						N252K	N248D
			Q245R	N248D	L257V	N248D	Q245R	Q245R	Q245R	N248D	N252K	N252K	N252K	N252K	N252K	N248D	Q245R
Q245R	Q245R		Q236H	Q245R	Q245R	Q245R	Q236H	Q236H	Q236H	Q245R	N248D	N248D	N248D	N248D	N248D	Q245R	Q236H
Ф236Н	Q236H	Q245R	A232V	Q236H	Q236H	Q236H	A232V	A232V	A232V	Q236H	Q245R	Q245R	Q245R	Q245R	Q245R	Q236H	A232V
A232V	A232V	Q236H	Y209W	A232V	A232V	A232V	S212G	S212G	S212G	A232V	Q236H	Q236H	Q236H	V244T	V244A	A232V	T213R
P210I	G159D	A230V	G159D	G159D	G159D	S212G	G159D	G159D	G159D	T213R	A232V	A232V	A232V	Q236H	Q236H	T213R	G159D
V205I	S128L	G159D	V104I	V104I	V104I	G159D	V104I	V104I	V104I	G159D	G159D	N184S	N184G	A232V	A232V	G159D	V104I
G159D	V104I	V104I	S103A	S103A	S103A	V104I	S103A	S103A	S103A	V104I	P131V	G159D	G159D	G159D	G159D	V1041	S103A
V104I	S103A	S103A	V68A	V68A	V68A	S103A	G102A	G102A	G102A	S103A	V104I	V104I	V104I	V104I	V104I	S103A	N62D
S103A	V68A	A48V	A48V	A48V	A48V	G102A	Q12R	S101G	A98L	G102A	S103A	S103A	S103A	S103A	S103A	N62D	Q12R

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									N252K								
					N252K		N252K	N252K	N248D			N252K				N252K	N252K
N252K	N252K	N252K	N252K	N252K	N248D	N252K	N248D	N248D	Q245R			N248D	N252K	N252K	N252K	N248D	N248D
N248D	N248D	N248D	N248D	N248D	Q245R	N248D	Q245R	Q245R	Q236H			Q245R	N248D	N248D	N248D	Q245R	Q245R
Q245R	Q245R	Q245R	Q245R	Q245R	Q236H	Q236Н	Q236H	Q236H	A232V			Q236H	Q245R	Q245R	Q245R	Q236H	Q236H
Ф236Н	Q236H	Q236H	Q236H	Q236H	A232V	A232V	A232V	A232V	T213R	N252K		A232V	Q236H	Q236H	Q236Н	A232V	A232V
A232V	A232V	A232V	A232V	A232V	S212G	S212G	T213R	T213R	S212G	N248D		T213R	A232V	A232V	A232V	T213R	T213R
N185D	Q206E	T213Q	G159D	G159D	G159D	G159D	G159D	S212G	G159D	Q245R	Q245R	G159D	G159D	G159D	G159D	G159D	G159D
G159D	G159D	G159D	V104I	V104I	V104I	V104I	Q109R	G159D	V104I	A232V	A230V	S130G	S130G	S128G	S128L	V104I	S128G
V104I	V104I	V104I	S103A	S103A	S103A	S103A	V104I	V104I	S103A	G159D	G159D	V104I	V104I	V104I	V104I	S103A	V104I
S103A	S103A	S103A	G102A	G102A	G102A	G102A	S103A	S103A	S101G	V104I	V104I	S103A	S103A	S103A	S103A	S101G	S103A
S101G	S101G	S101G	A98L	S101G	A98L	A98L	N62D	N62D	N62D	S103A	S103A	N62D	S101G	S101G	S101G	N62D	N62D

													E271Q
													N252K
N252K											N252K		N248D
N248D	T260A	N252K	N252K	N252K	N252K	N252K	N252K	N252K		N252K	N248D	N252K	Q245R
Q245R	N252K	N248D	N248D	N248D	N248D	N248D	N248D	N248D		N248D	Q245R	N248D	Q236H
T213R A232V Q236H	N248D	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R		Q245R	Q236H	Q245R	A232V
A232V	Q245R	Q236H	Q236H	Q236H	Q236H	Q236H	Q236H	Q236H	Q245R	Q236 Н	A232V	Q236H	T213R
T213R	Q236H	A232V	A232V	A232V	A232V	A232V	A232V	A232V	Q236H	A232V	A194P	A232V	Q206E
G159D	A232V	G159D	G159D	G159D	S212G	Y209W	P210I	V205I	A230V	A194P	G159D	A230V	N185D
S128L	G159D	P131V	V104I	V104I	G159D	G159D	G159D	G159D	G159D	G159D	V104I	G159D	G159D
V104I	V104I	V104I	S103A	S103A	V104I	V104I	V104I	V104I	V104I	V104I	S103A	V104I	V104I
S103A	S103A	S103A	S101G	S101G	S103A	S103A	S103A	S103A	S103A	S103A	S101G	S103A	S103A
N62D	S101G	S101G	A98V	S99G	S101G	S101G	S101G	S101G	S101G	S101G	N76D	S101G	N62D

Example 2

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A large number of the protease variants produced in Example 1 were tested for performance in two types of detergent and wash conditions using a microswatch assay described in "An improved method of assaying for a preferred enzyme and/or preferred detergent composition", U.S. Serial No. 60/068,796.

Table 5 lists the variant proteases assayed and the results of testing in two different detergents. For column A, the detergent was 0.67 g/l filtered Ariel Ultra (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 3 grains per gallon mixed Ca²⁺/Mg²⁺ hardness, and 0.3 ppm enzyme was used in each well at 20°C. For column B, the detergent was 3.38 g/l filtered Ariel Futur (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 15 grains per gallon mixed Ca²⁺/Mg²⁺ hardness, and 0.3 ppm enzyme was used in each well at 40°C.

able 5

В		1.11	1.85	1.20	1.67	1.42	1.80	1.78	1.34	1.67	0.53	0.20	1.41	0.47	1.28	0.09
A	-	0.56	1.41	2.77	2.26	2.96	1.91	2.05	2.00	2.38	2.83	2.87	2.56	3.97	3.35	3.77
																N252K
				N252K		N252K	N252K		N252K					R275H	L257V	N248D
			N252K	N248D		Q245R	Q245R	Q245R	Q245R	L257V	N248D	Q245R	N252S	L257V	Q245R	Q245R
			Q245R	Q245R	Q245R	Ф236Н	Q236 Н	Q236H	Q236H	Q245R	Q245R	K237E	Q245R	Q245R	Q236H	Q236H
			Q236H	Q236Н	Q236H	A232V	A232V	A232V	A232V	Q236H	Q236H	Q236H	Q236H	Q236H	A232V	A232V
			A232V	A232V	A232V	G159D	G159D	G159D	G159D	A232V	A232V	A232V	A232V	A232V	T224A	G159D
			G159D	G159D	G159D	N140D	V104I	V104I	V104I	G159D	G159D	G159D	G159D	G159D	G159D	V104I
	V104I	A228T	V104I	V104I	V104I	V104I	S103A	S103A	S103A	V104I	V104I	V104I	V104I	V104I	V104I	S103A
	S103A	V104I	S103A	S103A	S103A	S103A	V68A	V68A	V68A	S103A	S103A	S103A	S103A	S103A	S103A	V68A
	N76D	S103A	V68A	V68A	V68A	V68A	N43S	N43K	N43D	V68A	V68A	V68A	V68A	V68A	V68A	G61E

0.47	1.46	0.28	0.33	0.36	0.43	0.32	0.33	0 13	0.35	0.55	0.25	0.48	0.19	0.29	0.53	0.12	0.43
3.50	2.81	1.56	1.22	1.13	1.22	1.12	1.54	1.04	1.09	1.11	1.50	1.11	1.05	1.32	1.19	0.92	1.31
N252K	N252K																
N248D	N248D																
Q245R	Q245R																
Ф236Н	Q236H																
G159D A232V	A232V																
G159D	S212P				N248D				A174V	K237Q							
V104I	G159D	V104I	V1041	V104I	V104I	V104I	N261D	S216C	V104I	V104I	N183D	V104I	V104I	N184D	N252D	S259C	K251T
S103A	V104I	S103A	S103A	S103A	S103A	S103A	V104I	V104I	S103A	S103A	V1041	S103A	S103A	V104I	V104I	V104I	V104I
V68A	S103A	A98E	N76D	N77D	D9ZN	U36D	S103A	S103A	DZZN	N76D	S103A	N76D	N76D	S103A	S103A	S103A	S103A
N43D	V68A	D9ZN	V4E	N76D	A16T	A1E	N76D	N76D	U36D	T38S	N76D	R19L	R19C	U36D	N76D	N76D	U36D

0.98	0.37	0.16	0.99	0.23	0.28	0.71	1.26	0.87	1.07	1.31	1.35	1.02	0.92	1.25	1.32	1.10	1.17
1.00	1.70	1.12	1.13	1.88	1.29	0.52	0.23	0.21	0.24	0.61	0.69	0.37	0.98	0.63	0.49	0.39	0.34
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																N183I	
	N185D	T274A			K237E			N204T			E271V	N261Y			S242T	N116K	
V104I	V104I	K237E	A228V	G159D	V104I	S130L	Q109R	V104I	D181N	V104I	S212P	N252K	S242T	E271Q	V104	V104I	G258R
S103A	S103A	V104i	V104I	V104I	S103A	V104I	V104I	S103A	V104I	S103A	V104I	V104I	V104I	V104I	S103A	S103A	V104I
P86S	U36D	S103A	S103A	S103A	N76D	S103A	S103A	S99R	S103A	N76D	S103A	S103A	S103A	S103A	N76D	N76D	S103A
M76D	1727	N76D	N76D	N76D	H17L	N76D	N76D	N76D	N76D	Q12R	N76D	N76D	N76D	N76D	Q12R	N43S	N76D

1.25	0.95	0.98	0.91	1.02	1.01	1.02	1.06	1.26	0.04	0.05	0.04	0.16	0.88	0.03	0.04	0.04	0.04
0.57	0.22	0.24	0.13	0.16	0.31	0.33	0.38	0.84	1.97	1.51	1.40	1.95	2.41	1.34	1.78	2.16	1.91
					-		-		-	_		-					
														K251T			
														N185D		V244A	
	1198V	Q182R	Q137R	N248S	Q206R		G258R	E271G	N261D	Q206E	Q206E			A133T	N261D	Q206E	S188E
E271G	Q182R	V104I	M1191	Q137R	V104I	Q206R	S212P	V104I	Q206E	V104I	V104I	A158E	Q206E	V104I	Q206E	G159D	V104I
V104I	V104I	S103A	V104I	V104I	S103A	V104I	V104I	S103A	V104I	S103A	S103A	V104I	V104I	S103A	V104I	V104I	S103A
S103A	S103A	N76D	S103A	S103A	U36D	S103A	S103A	D9/N	S103A	N76D	U77D	S103A	S103A	DZZN	S103A	S103A	N76D
M76D	N76D	L21M	N76D	N76D	A13T	N76D	N76D	T58S	N76D	V4E	N76D	N76D	N76D	N76D	N76D	N76D	V4E

0.04	90.0	0.16	0.09	0.17	0.14	0.18	0.19	0.15	0.07	1.42	2.03	1.79	1.78	1.21	0.78	0.44	0.45
2.06	1.73	2.04	3.20	1.83	1.42	1.86	1.87	1.90	1.61	0.44	0.39	0.62	0.11	0.12	1.63	2.37	2.97
						 	-										
					-				-					-			
																	E271V
		G159D	Ф236Н		G159D					E271V	E271V	E271V					Q236H
A158E	K251T	L111M	G159D	G159D	V104I	G159D	G159D	N238S	T224A	V268F	S212P	Q245L	Q245R	P210L	V104I	Q236H	G159D
V104I	Q206E	V104I	V104I	V104I	S103A	V104I	G146S	G159D	G159D	S212P	V104I	S212P	Q109R	Q109R	S103A	V104I	V104I
S103A	V104I	S103A	S103A	S103A	N76D	S103A	V104I	V104	V104I	V104I	S103A	V104I	V104I	V104I	N76D	S103A	S103A
M76D	S103A	M76D	Q9ZN	09/N	N62H	N76D	S103A	S103A	S103A	S103A	S87R	S103A	S103A	S103A	N62S	N76D	Q9/N
V4E	N76D	A48T	V68A	L42V	Q12H	L42I	N76D	N76D	N76D	N76D	N76D	N76D	N76D	N76D	G20V	V68A	V68A

09ZN	S103A	V104I	G159D	Q236 Н	Q245R			3.00	0.61
100	S103A	V104I	G159D	L217I	Q236H	E271V		2.71	0.12
	N76D	S103A	V104I					2.46	0.38
	S103A	V104I						2.46	0.61
	S103A	V104I	G159D	Q236R				3.31	0.11
+	N76D	S103A	V104I	G159D	Q236 Н			3.06	0.14
+	S103A	V104I	A114V	V121I	G159D	Q236Н	Q245R	3.11	0.40
+	Q92N	S103A	V104I	G159D	Q236H			3.12	0.34
+	S103A	V104I	G159D	Y209S	Q236H	T253K		3.18	0.03
N76D	S103A	V104I	N117K	G159D	N184S	Ф236Н		2.78	90.0
M76D	S103A	V104I	Ф236Н					2.49	0.57
M76D	S103A	V104I	G159D	Q236H	Q245L			3.37	0.03
N76D	S103A	V104I	N123S	G159D	Q236H	H249Y		3.11	0.03
M76D	S103A	V104I	G159D	Q236H	H249Q			3.15	0.04
N76D	S103A	V104I	G159D	Q236H	Q245R	N261D		3.31	0.03
M76D	S103A	V104I	S141N	G159D	Q236H	Q245R	T255S	3.26	0.62
N76D	S103A	V104I	G159D	Q236H	Q245R	R247H		2.78	0.03
N76D	S103A	V104I	G159D	A174V	N204D	Q236H	Q245R	3.28	0.02

0.02	0.03	0.58	0.13	1.73	1.13	1.54	0.8	1.5	0.15	1.09	0.99	1.76	1.06	1.92	1.45	1.72	1.59
3.34	3.28	2.91	2.86	1.30	1.83	1.28	3.72	9.0	1.91	1.92	3.57	1.74	3.15	2.33	1.67	2.16	2.77
				T260A										Q245R			S259G
	Q245R		Q245R	Q245R		Q245R	L257V				Q245R	Q245R	Q245R	Q236H	Q245R	Q245R	Q245R
Q245R	Q236Н	Q245R	Ф236Н	Q236H		Q236Н	Q245R			L257V	Q236 Н	Q236Н	Q236Н	A232V	Q236H	д 236Н	Q236H
Q236 Н	N218D	Q236Н	V203A	A232V		A232V	Q236H	L257V		Q245R	A232V	A232V	A232V	Y214L	A232V	A232V	A232V
N204D	G159D	A232V	A1941	T213R		P210R	A232V	Q245R		Q236 Н	Y209W	G211R	G211V	G159D	A215R	G159D	G159D
G159D	A133V	G159D	G159D	G159D	V104I	G159D	G159D	Q236H	R275H	A232V	G159D	G159D	G159D	V104I	G159D	V104I	V104I
V104I	V104I	V104I	V104I	V104I	S103A	V104I	V104I	A232V	L257V	G159D	V104I	V104I	V104I	S103A	V104I	S103A	S103A
S103A	S103A	S103A	S103A	S103A	N76D	S103A	S103A	V104I	V104I	V104I	S103A	S103A	S103A	U92N	S103A	N76D	Q9/N
Q9/N	Q9/N	N76D	M76D	M76D	V68A	N76D	M76D	S103A	S103A	S103A	M76D	N76D	U36D	V68A	N76D	V68A	V68A
V68A	V68A	V68A	V68A	V68A	T22K	V68A	V68A	Q9/N	N76D	N76D	V68A	V68A	V68A	Q12R	V68A	Q12R	G20R

1.49	0.68	1.37	1.2	0.76	1.86	1.44	1.14	1.29	1.81	1.53	1.72	1.62	1.08	Q	1.23	1.65	0.46
2.62	2.92	2.17	0.48	2.92	2.09	0.51	1.60	1.35	1.92	1.17	2.01	2.09	3.00	S	1.01	0.57	0.86
												_		Q245R			
T260V					Q245R			K251R	A272S	Q245R				Q236H			
Q245R	N261G	N261W		Q245R	Q236H			N248S	Q245R	Q236Н	S256R	Q245R	Q245R	A232V			
Q236 Н	Q245R	Q245R		Q236Н	A232V		Q245R	Q245R	Q236H	A232V	Q245R	Q236H	Q236H	N185S			
A232V	Q236H	Q236H	Q245R	A232V	G159D		Q236H	Q236H	A232V	Q206L	Q236H	A232V	A232V	R170S			
G159D	A232V	A232V	S242P	P210L	V104I	Q245R	A232V	A232V	G159D	N183K	A232V	Q206R	G159D	G159D		H249R	
V104I	G159D	G159D	Q236H	G159D	S103A	Q236H	Y192F	G159D	V104I	G159D	G159D	G159D	V104I	N116T	Q245R	M222S	M222S
S103A	V104I	V104I	A232V	V104I	U36D	A232V	G159D	V147I	S103A	V104I	V104I	V104I	S103A	V104I	M222S	V104I	N173R
S87R	S103A	S103A	V104I	S103A	V68A	V104I	V104I	V104I	N76D	S103A	S103A	S103A	N76D	S103A	V104I	S103A	V104I
N76D	Q9/N	N76D	S103A	N76D	A48V	S103A	S103A	S103A	V68A	M76D	N76D	N76D	V68A	N76D	S103A	N76D	S103A
V68A	V68A	V68A	M76D	V68A	Q12R	N76D	N76D	N76D	Q12R	V68A	V68A	V68A	K27R	V68A	N76D	Q12R	N76D

0.77	0.76	1.16	1.12	96.0	1.25	1.01	1.46	1.56	1.74	1.56	1.61	1.85	1.56	1.30	1.30	0.16	0.04
1.24 (1.18	0.52	0.56	0.43	0.42	1.15	0.53	0.69	0.66	0.52	0.70	0.79	0.78	1.25	1.29	1.44	2.01
-	-	0	0	0	0	_	0	0	0	0	0	0	0	-	-	-	2
																	Q245R
													L262S			K251Q	N243D
												Q245R	Q245R	N261D		Q245R	M222S
	Y263F								Q245R	Q245R	Q245R	M222S	V227A	Q245R		M222S	N185D
	K237R		E271D				Q245R		V244I	P210T	M222S	A215V	M222S	M222S	Q245R	S130T	R170S
Y263F	M222S	M222S	M222S	M222S	M222S	H249R	M222S	Q245R	M222S	M222S	S130T	S130T	S130T	S130T	M222S	1104T	S130T
M222S	V104I	Q109R	Q109R	V104I	Q137R	M222S	V104I	A232V	1104T	V104I	1104T	1104T	1104T	1104T	S130T	S103A	1104T
V104I	S103A	V104I	V104I	S103A	V104I	V104I	S103A	V104I	S103A	S103A	S103A	S103A	S103A	S103A	1104T	N76D	S103A
S103A	Q92N	S103A	S103A	N76D	S103A	S103A	N76D	S103A	09ZN	Q9/N	Q9/N	M76D	N76D	N76D	S103A	S57P	N76D
N76D	L21M	N76D	N76D	G61R	N76D	N76D	Q12R	N76D	Q12R	Q12R	Q12R	Q12R	Q12R	Q12R	N76D	Q12R	Q12R

0.77 1.60	0.73 1.66	2.09 0.86
0	0	2
V268A	Q245R	Q245R
Q245R	P210S	Q236Н
M222S	M222S	A232V
S103A 1104T S130T M222S Q245R V268A	S103A 1104T S130T M222S P210S Q245R	S103A V104I G159D A232V Q236H Q245R
1104T	1104T	V104I
S103A	S103A	S103A
N76D	N76D	U36D
Q12R	Q12R	V68A

Example 3

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Table 6 lists the variant proteases assayed from Example 1 and the results of testing in four different detergents. The same performance tests as in Example 2 were done on the noted variant proteases with the following detergents. For column A, the detergent was 0.67 g/l filtered Ariel Ultra (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 3 grains per gallon mixed Ca²⁺/Mg²⁺ hardness, and 0.3 ppm enzyme was used in each well at 20°C. For column B, the detergent was 3.38 g/l filtered Ariel Futur (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 15 grains per gallon mixed Ca²⁺/Mg²⁺ hardness, and 0.3 ppm enzyme was used in each well at 40°C. For column C, 3.5g/l HSP1 detergent (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 8 grains per gallon mixed Ca²⁺/Mg²⁺ hardness, and 0.3 ppm enzyme was used in each well at 20°C. For column D, 1.5 ml/l Tide KT detergent (Procter & Gamble, Cincinnati, OH, USA), in a solution containing 3 grains per gallon mixed Ca²⁺/Mg²⁺ hardness, and 0.3 ppm enzyme was used in each well at 20°C.

Table 6

۵	-	1.26	2.35	1.19	1.31	2.02	2.70	0.80	2.88	1.78	2.07	2.01	2.66	2.78	0.75
၁	-	1.39	1.65	1.20	1.66	1.60	1.48	1.23 (1.41	1.55	1.63	1.62	1.36	1.27	1.31
8	-	<u> </u>	1	<u> </u>		1	<u>l</u>	L	1	1	1	1	l	ŀ	i i
		1.41	1.49	1.41	1.72	1.38	0.91	1.39	0.86	1.43	1.43	1.47	0.56	0.50	1.38
A		1.44	2.34	1.05	1.81	2.19	2.91	0.93	2.67	2.22	2.30	2.31	2.63	2.75	1.11
													<u> </u>		
									N252K						
			N252K	N252K	N252K	N252K	N252K	N252K	N248D	N252K	N252K	N252K	N252K	N252K	
			N248D	N248D	N248D	N248D	N248D	N248D	Q245R	N248D	N248D	N248D	N248D	N248D	N252K
		N252K	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	Q236H	Q245R	Q245R	Q245R	Q245R	Q245R	N248D
	••	N248D	Q236 Н	Ф236Н	Ф236Н	Q236Н	Q236H	Q236 Н	A232V	Q236 Н	Q236H	Q236H	Q236H	Q236Н	Q245R
		Q245R	A232V	A232V	A232V	A232V	A232V	A232V	P210L	A232V	A232V	A232V	A232V	A232V	Q236H
		Q236H	Y209W	G159D	G159D	Y209F	N185D	P210R	N185D	P210L	S212C	S212G	\$212E	T213E	A232V
		A232V	G159D	Q109R	V104I	G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	T213S
	V104I	G159D	V104I	V104I	S103A	V104I	V104I	V104I	V104I	V104I	V104I	V104I	V104I	V104I	V104I
	S103A	V104I	S103A	S103A	V68A	S103A	S103A	S103A	S103A	S103A	S103A	S103A	S103A	S103A	S103A
	N76D	S103A	V68A	V68A	G20R	V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A

2 2.01	1.06	3 1.54	1.20	1.56	1.87	2.89	2.42	0.95	2.42	1.85	3.22	3 1.72	1.65	3 2.58	0.94	1.05	1.18
1.12	1.37	1.53	1.47	1.56	1.47	1.07	1.29	1.24	1.42	1.30	1.43	1.58	1.59	1.33	1.46	1.31	0.85
0.15	1.42	1.40	1.58	1.36	1.36	0.33	0.46	1.46	1.00	1.13	0.91	1.36	1.46	0.77	1.52	1.41	1.41
2.27	1.37	2.14	1.22	2.12	1.88	2.24	2.43	0.98	2.52	2.05	2.61	2.18	2.14	2.46	1.31	1.21	1.51
												ļ <u>-</u>					
N252K	N252K	N252K	N252K	N252K	N252K	N252K	N252K	N252K			N252F	T255V	S256N	S256E	S256R	T260R	L257R
N248D	N248D	N248D	N248D	N248D	N248D	N248D	N248D	N248D	N252F	N252L	N248D	N252K	N252K	N252K	N252K	N252K	N252K
Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	N248D	N248D	Q245R	N248D	N248D	N248D	N248D	N248D	N248D
Q236Н	Q236H	Q236H	Q236H	Q236H	Ф236Н	Ф236Н	Ф236Н	Q236H	Q245R	Q245R	Q236H	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R
A232V	A232V	A232V	A232V	A232V	A232V	A232V	A232V	A232V	Ф236Н	Ф236Н	A232V	Ф236Н	Q236H	Q236H	Q236H	Q236H	Q236H
T213E	T213R	A215V	A215R	S216T	S216V	S216C	N173D	Q206R	A232V	A232V	G159D	A232V	A232V	A232V	A232V	A232V	A232V
G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	V104I	G159D	G159D	G159D	G159D	G159D	G159D
V104I	V104I	V104	V104I	V104I	V104I	V104I	V104I	V104I	V104I	V104I	S103A	V104I	V104I	V104I	V104I	V104I	V104I
A103V	S103A	S103A	S103A	S103A	S103A	S103A	S103A	S103A	S103A	S103A	V68A	S103A	S103A	S103A	S103A	S103A	S103A
V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A	V68A	P55S	V68A	V68A	V68A	V68A	V68A	V68A

2.64	0.84	0.73	2.67	1.57	2.44	2.29	1.27	1.56	1.15	1.28	2.25	1.28	1.45	1.55	1.40	1.72	1.71
1.30	1.37	1.32	1.41	1.53	1.33	1.36	0.89	1.62	1.67	1.11	1.43	S S	S S	2	N Q	Q.	ND .
0.59	1.47	1.50	0.93	1.38	0.25	0.97	1.54 (1.50	1.72	1.30	0.83	0.07	09.0	0.79	0.41	0.68	0.68
2.56 0	1.02	1.04	2.60 0	<u>. </u>	2.83 0	2.10 0	1.37	2.30 1	1.72 1	1.32	2.50 0	₩	+	┼		├	 i
2.5	-	-	5	2.31	5	7	-	2	-	-	2.	4.20	3.47	4.32	3.14	2.71	2.97
							ļ			T260A							
				<u> </u>	X					-		놋	ᄎ -	ᄎ	ļ	-	
					N252K					Q245R		N252K	N252K	N252K			
G258D	N261R			N252K	N248D	N252K		N252K	N252K	О236Н	N252K	N248D	N248D	N248D			
N252K	N252K		N252K	N248D	Q245R	N248D	N252K	N248D	N248D	A232V	N248D	Q245R	Q245R	Q245R	N252K	N252K	N252K
N248D	N248D	N252K	N248D	Q245R	Ф236Н	Q245R	N248G	Q245R	Q245R	T213R	Q245R	Q236H	Q236H	Q236 Н	N248D	N248D	N248D
Q245R	Q245R	N248D	Q245V	Ф236Н	A232V	Q236H	Q245R	О236Н	Ф236Н	P210L	О236Н	A232V	A232V	A232V	Q245R	Q245R	Q245R
Ф236Н	Ф236Н	Q245R	Ф236Н	A232V	G159D	A232V	Ф236Н	A232V	A232V	G159D	A232V	S160V	V104I	Y167F	О236Н	Ф236Н	Ф236Н
A232V	A232V	Д236Н	A232V	A228V	S130A	G159D	A232V	N218S	G159D	V104I	G159D	G159D	S103A	G159D	A232V	A232V	A232V
G159D	G159D	A232V	G159D	G159D	V104I	A133V	G159D	G159D	V104I	S103A	V104I	V104I	U36N	V104I	G159D	G159D	G159D
V104I	V104I	V104I	V104I	V104I	S103A	V104I	V104I	V104I	S103A	E89D	S103A	S103A	V68A	S103A	V104I	V104I	V104I
S103A	S103A	S103A	S103A	S103A	V68A	S103A	S103A	S103A	V68A	N76D	U26D	V68A	G61E	V68A	S103A	S103A	S103A
V68A	V68A	V68A	V68A	V68A	G61E	G61E	V68A	V68A	G20R	V68A	V68A	G61E	S3L	G61E	G97E	A98D	S99E

8	1.33	1.69	7	ę	ထွ	22	စ္	72	75	ල	စ္က	72	1.10	2.55	2.40	1.86	1.95
1.90		_	2.71	2.40	2.58	1.82	2.46	2.84	3.97	3.09	2.60	2.54		_	-	 	\vdash
2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
0.27	1.80	1.33	0.55	1.05	2.19	2.16	0.13	1.36	1.21	0.95	2.83	1.92	2.61	2.46	2.08	2.04	2.11
3.50	2.24	3.35	4.88	4.22	5.45	3.76	7.42	5.43	5.12	6.38	3.17	4.38	3.05	4.09	2.32	2.34	2.24
											N252K			-			
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											N248D	N252K	N252K	N252K			
N252K	N252K	N252K	N252K	N252K	N261R	N252K	N252K	N252K	N252K	N252K	Q245R	N248D	N248D	N248D	N252K	N252K	N252K
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N248D	N248D	N248D	N248D	N248D	N252K	N248D	N248D	N248D	N248D	N248D	Q236H	Q245R	Q245R	Q245R	N248D	N248D	N248D
Q245R	Q245R	Q245R	Q245R	Q245R	N248D	Q245R	Q245R	Q245R	Q245R	Q245R	A232V	Q236H	Q236H	Q236Н	Q245R	Q245R	Q245R
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Q236H	Q236H	Q236H	Q236H	Q236H	Q245R	Q236H	Q236H	Q236H	Q236H	Q236H	T213R	A232V	A232V	A232V	Q236H	V244T	V244A
A232V	A232V	A232V	A232V	A232V	Q236H	A232V	A232V	A232V	A232V	A232V	G159D	T213R	L217E	Q206R	A232V	Q236H	Q236H
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G159D	G159D	G159D	G159D	G159D	A232V	G159D	G159D	N184D	S166D	L217E	V104I	G159D	Q206R	G159D	N184G	A232V	A232V
V104	V104	V104I	S106E	Q109E	G159D	Q109R	V104I	G159D	G159D	G159D	S103A	V104	G159D	V104I	G159D	G159D	G159D
<u> </u>	┼	+	+-	┼	+	+-	+-	╅	+	+	+	+	+-	+-	+	+-	+
S103A	S103A	S103A	V104I	V104I	V104I	V104I	S103A	V104	V104	V104	N62D	S103A	V104I	S103A	V104	V104I	V104I
S101E	S101G	G102A	S103A	S103A	S103A	S103A	N62D	S103A	S103A	S103A	G20R	N62D	S103A	N62D	S103A	S103A	S103A
S	S	હ	S	လ	လ	S	Ž	S	လ	S	Ø	Ž	တ	Ž	S	S	လ

2.47	1.82	1.44	1.99	5.39	1.92	1.36	1.01	2.88	3.84	3.19	2.17	2.25	2.08	2.25	2.34	1.86	1.49
S	Q	2	Q.	2	Q	Q	QN	Q N	QN	Q	QN	Q	QN	Q	Q	Q	QN
1.56	2.09	2.66	2.78	0.94	1.41	0.57	1.86	0.50	1.20	2.10	2.67	0.41	2.07	2.48	2.76	2.10	2.35
2.81	2.30	2.63	2.01	7.74	5.14	4.97	2.41	4.42	5.86	5.87	2.98	4.02	6.63	2.03	2.96	2.74	2.11
				E2710													
				N252K													
		S256R	N252K	N248D							N252K	N252K	N252K		N252K		
		N252K	N248D	Q245R	N252K	N252K	N252K	N252K	N252K	N252K	N248D	N248D	N248D	N252K	N248D		
N252K	N252K	N248D	Q245R	Q236H	N248D	N248D	N248D	N248D	N248D	N248D	Q245R	Q245R	Q245R	N248D	Q245R		
N248D	N248D	Q245R	Q236H	A232V	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	Q236H	0236Н	Q236H	Q245R	Q236H		
Q245R	Q245R	Ф236Н	A232V	T213R	Q236 Н	О236Н	Q236H	О236Н	Q236H	Ф236Н	A232V	A232V	A232V	Q236 Н	A232V	N252K	
Ф236Н	Ф236Н	A232V	T213R	Q206E	A232V	A232V	A232V	A232V	A232V	A232V	S212G	S212G	S212G	A232V	T213R	N248D	
A232V	A232V	T213R	G159D	N185D	N185D	Q206E	T213Q	G159D	G159D	S212G	G159D	G159D	G159D	T213R	G159D	Q245R	Q245R
G159D	G159D	G159D	V104I	G159D	G159D	G159D	G159D	V104I	V104I	G159D	V104I	V104I	V104I	G159D	Q109R	A232V	A230V
V104I	V104I	V104I	S103A	V104I	V104I	V104I	V104I	S103A	S103A	V104I	S103A	S103A	S103A	V104I	V104I	G159D	G159D
S103A	S103A	S103A	N62D	S103A	S103A	S103A	S103A	G102A	G102A	S103A	G102A	G102A	G102A	S103A	S103A	V104I	V104I
K27N	T38G	N62D	Q12R	N62D	S101G	S101G	S101G	A98L	S101G	G102A	Q12R	A98L	S101G	G102A	N62D	S103A	S103A

2.58	1.61	0.6	1.08	2.35	1.77	1.45	3.05	1.08	1.20	1.01	8.7	1.03	1.05	1.23	1.10	1.25	2
S S	N 0	Q	8	QN QN	S S	8	S S	S S	N ON	N Q	QN	N Q	N Q	Q.	2	2	128
0.71	1.32	1.23	0.71	0.83	1.38	0.07	1.16	1.34	1.47	1.38	1.18	1.23	1.38	121	1.30	0.80	,
3.42 0	2.59	1.30	2.94 0	3.17 0	2.15	3.07 0	2.26 1	1.82	2.16	1.79 1	1.15 1	1.47	1.90	1.55 1	1.96	2.49	420
3	2	-	2	3	2	3	2	-	2	-	-	-	-			2	4
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N252K				N252K	N252K	N252K										N252K	N252K
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N248D	N252K	N252K	N252K	N248D	N248D	N248D	N252K	N252K	N252K	N252K	N252K	N252K	N252K		N252K	N248D	N248D
Q245R	N248D	N248D	N248D	Q245R	Q245R	Q245R	N248D	N248D	N248D	N248D	N248D	N248D	N248D		N248D	Q245R	Q245R
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Q236H	Q245R	Q245R	Q245R	Q236H	Q236H	Q236H	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R		Q245R	Q236H	Q236H
A232V	Q236H	Q236H	Q236H	A232V	A232V	A232V	Q236H	Q236H	Q236Н	Q236H	Q236Н	Q236Н	Q236H	Q245R	Q236H	A232V	A232V
I3R A	╁	-	┼	I3R A	3R A	3R A	+	32V Q	32V Q	┿	┼	+	╁	 	i	+	60V A
T213	A232V	A232V	A232V	T213	T213	T213	A232V	A232	A232	A232V	A232V	A232V	A232V	Q236H	A232V	A194P	S160
G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	S212G	Y209W	P210I	V205I	A230V	A194P	G159D	G159D
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S130G	S130G	S128G	S128L	V104I	S128G	S128L	P131V	V104	V104	G159D	G159D	G159D	G159D	G159D	G159D	V104	V104
V104I	V104I	V104I	V104I	S103A	V104I	V104I	V104I	S103A	S103A	V104I	V104I	V104I	V104I	V104I	V104I	S103A	S103A
S103A	S103A	S103A	S103A	S101G	S103A	S103A	S103A	S101G	S101G	S103A	S103A	S103A	S103A	S103A	S103A	S101G	V68A
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N62D	S101G	S101G	S101G	N62D	N62D	N62D	S101G	A98V	S99G	S101G	S101G	S101G	S101G	S101G	S101G	N76D	G61E

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QN	Q.	Q.	<u>Q</u>	Q	QN	QN	QN	Q.	QN	Q	QN	QN	QN	QN	Q.	S	<u>Q</u>
145	155	140	172	171	190	133	169	271	240	258	182	246	284	397	309	260	254
09	79	41	89	89	27	180	133	55	105	219	216	13	136	121	92	283	192
347	432	314	271	297	350	224	335	488	422	545	376	742	543	512	638	317	438
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<u>.</u>												-			-		
N252K	N252K								:							N252K	
N248D	N248D															N248D	N252K
	-	×	×	Y	X	×	×	×	Y	2	×	×	노	노	노	₩	
Q245R	Q245R	N252K	N252K	N252K	N252K	N252K	N252K	N252K	N252K	N261R	N252K	N252K	N252K	N252K	N252K	Q245R	N248D
Ф236Н	Q236H	N248D	N248D	N248D	N248D	N248D	N248D	N248D	N248D	N252K	N248D	N248D	N248D	N248D	N248D	Ф236Н	Q245R
A232V (A232V (Q245R 1	Q245R 1	Q245R 1	Q245R	Q245R 1	Q245R 1	Q245R 1	Q245R 1	N248D	Q245R 1	Q245R	Q245R 1	Q245R	Q245R	A232V (0236Н (
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V104I	Y167F	Q236H	О236Н	Q236H	Q236H	Q236H	О236Н	Q236H	Q236H	Q245R	Q236H	Q236H	Q236H	Q236H	О236Н	T213R	A232V
S103A	G159D	A232V	A232V	A232V	A232V	A232V	A232V	A232V	A232V	Ф236Н	A232V	A232V	A232V	A232V	A232V	G159D	T213R
-	┼	+-	+	+	+	1	+	+	+	1.	+	+	+	+	+-	+	+
N76D	V104	G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	A232V	G159D	G159D	N184D	S166D	L217E	V104I	G159D
V68A	S103A	V104I	V104I	V104I	V104I	V104I	V104I	S106E	Q109E	G159D	Q109R	V104I	G159D	G159D	G159D	S103A	V104I
⊢	+	+	┼	┼	┼	┼	╬	+	+	+	+-	┿	1-	+	1	+	+-
G61E	V68A	S103A	S103A	S103A	S103A	S103A	S103A	V104I	V104I	V104I	V104I	S103A	V104	V104I	V104I	N62D	S103A
S3L	G61E	G97E	A98D	S99E	S101E	S101G	G102A	S103A	S103A	S103A	S103A	N62D	S103A	S103A	S103A	G20R	N62D

g	2	읒	ON N	S	Q Q	S S	2		N O	Q	Q.	Q.	Q Q	<u>S</u>	Q Q	2	Q.
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110	255	240	186	195	247	182	144	199	539	192	136	101	288	384	319	217	225
261	246	208	204	211	156	509	766	278	क्र	141	22	186	20	120	210	267	41
305	409	232	234	224	281	230	263	201	774	514	497	241	442	586	287	298	402
									E2710								
									N252K								
							S256R	N252K	N248D							N252K	N252K
N252K	N252K						N252K	N248D	Q245R	N252K	N252K	N252K	N252K	N252K	N252K	N248D	N248D
N248D	N248D	N252K	N252K	N252K	N252K	N252K	N248D	Q245R	Q236H	N248D	N248D	N248D	N248D	N248D	N248D	Q245R	Q245R
Q245R	Q245R	N248D	N248D	N248D	N248D	N248D	Q245R	Q236H	A232V	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R	Ф236Н	Q236H
Q236H	Q236H	Q245R	Q245R	Q245R	Q245R	Q245R	Q236H	A232V	T213R	Q236H	Q236H	Q236H	Q236H	Q236H	Q236H	A232V	A232V
A232V	A232V	Q236H	V244T	V244A	Q236H	Q236H	A232V	T213R	Q206E	A232V	A232V	A232V	A232V	A232V	A232V	S212G	S212G
L217E	Q206R	A232V	Q236H	Ф236Н	A232V	A232V	T213R	G159D	N185D	N185D	Q206E	T213Q	G159D	G159D	S212G	G159D	G159D
Q206R	G159D	N184G	A232V	A232V	G159D	G159D	G159D	V104I	G159D	G159D	G159D	G159D	V104I	V104I	G159D	V104I	V104I
G159D	V104I	G159D	G159D	G159D	V104I	V104I	V104I	S103A	V104I	V104I	V104I	V104I	S103A	S103A	V104I	S103A	S103A
V104I	S103A	V104I	V104I	V104I	S103A	S103A	S103A	N62D	S103A	S103A	S103A	S103A	G102A	G102A	S103A	G102A	G102A
S103A	N62D	S103A	S103A	S103A	K27N	T38G	N62D	Q12R	N62D	S101G	S101G	S101G	A98L	S101G	G102A	Q12R	A98L

2	QN	Q	Q	Q	Q	Q	QN	2	QN	Q	Q	Q	Q	Q	Q	2	QN
208	225	234	186	149	258	161	06	108	235	177	145	305	108	120	101	87	103
207	248	276	210	235	71	132	123	71	83	138	7	116	134	147	138	118	123
663	203	296	274	211	342	259	130	294	317	215	307	226	182	216	179	115	147
												-					
N252K		N252K			N252K				N252K	N252K	N252K				-		
	-	 	-		1	-	-	-	┿	-	 	~	~	~	~	Y	\ <u>\</u>
N248D	N252K	N248D			N248D	N252K	N252K	N252K	N248D	N248D	N248D	N252K	N252K	N252K	N252K	N252K	N252K
Q245R	N248D	Q245R			Q245R	N248D	N248D	N248D	Q245R	Q245R	Q245R	N248D	N248D	N248D	N248D	N248D	N248D
Q236H Q245R	Q245R	Q236H			Q236H	Q245R	Q245R	Q245R	Q236H	Ф236Н	Ф236Н	Q245R	Q245R	Q245R	Q245R	Q245R	Q245R
A232V	Q236H	A232V	N252K		A232V	Q236H	Q236H	Q236H	A232V	A232V	A232V	Q236H	Q236H	Q236H	Q236H	Q236H	Q236H
S212G	A232V	T213R	N248D		T213R	A232V	A232V	A232V	T213R	T213R	T213R	A232V	A232V	A232V	A232V	A232V	A232V
G159D	T213R	G159D	Q245R	Q245R	G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	G159D	S212G	Y209W	P210I
V104I	G159D	Q109R	A232V	A230V	S130G	S130G	S128G	S128L	V104I	S128G	S128L	P131V	V104I	V104I	G159D	G159D	G159D
S103A	V104I	V104I	G159D	G159D	V104I	V104I	V104I	V104I	S103A	V1041	V104I	V104I	S103A	S103A	V104I	V104I	V104I
S101G G102A	S103A	S103A	V104I	V104I	S103A	S103A	S103A	S103A	S101G	S103A	S103A	S103A	S101G	S101G	S103A	S103A	S103A
S101G	G102A	N62D	S103A	S103A	N62D	S101G	S101G	S101G	N62D	N62D	N62D	S101G	A98V	S99G	S101G	S101G	S101G

QN	QN	QN	QN
105	123	110	125
190 138 105 ND	155 151 123 ND	196 130 110 ND	249 80 125 ND
190	155	196	549
	:		
N252K		N252K	N252K
N248D		N248D	N248D
Q245R		Q245R	Q245R
Q236H	Q245R	232V Q236H Q245R N248D N252K	232V Q236H Q245R N248D N252K
A232V	Q236H	A232V	A232V
V205I	A230V	A194P	G159D
G159D	G159D	G159D	V104I
<u>2</u>	V10 <u>4</u>	V104	S103A
S101G S103A V104I G159D V205I A232V Q236H Q245R N248D N252K	S101G S103A V104I G159D A230V Q236H Q245R	S101G S103A V104I G159D A194P A	N76D S101G S103A V1041 G159D A
S101G	S101G	S101G	M76D

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WHAT IS CLAIMED:

- A protease variant comprising replacing an amino acid at one or more residue positions corresponding to residue positions selected from the group
 consisting of 62, 212, 230, 232, 252 and 257 of *Bacillus amyloliquefaciens* subtilisin.
 - 2. The protease variant according to claim 1 which is derived from a *Bacillus* subtilisin.
- 3. The protease variant according to claim 2 which is derived from *Bacillus* lentus subtilisin.
 - 4. A DNA encoding a protease variant of claim 1.
- 15 5. An expression vector encoding the DNA of claim 4.
 - 6. A host cell transformed with the expression vector of claim 5.
 - 7. A cleaning composition comprising the protease variant of claim 1.

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- 8. An animal feed comprising the protease variant of claim 1.
- 9. A composition for treating a textile comprising the protease variant of claim 1.

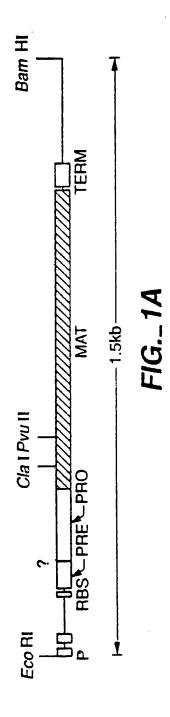
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- 10. The protease variant according to claim 1 comprising a substitution set selected from the group consisting of residue positions corresponding to positions in Table 1 of *Bacillus amyloliquefaciens* subtilisin.
- 30 11. The protease variant according to claim 10 comprising a substitution set selected from the group consisting of residue positions corresponding to positions in Table 2 of *Bacillus amyloliquefaciens* subtilisin.

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12. The protease variant according to claim 10 comprising a substitution set selected from the group consisting of residue positions corresponding to positions in Table 3 of *Bacillus amyloliquefaciens* subtilisin.



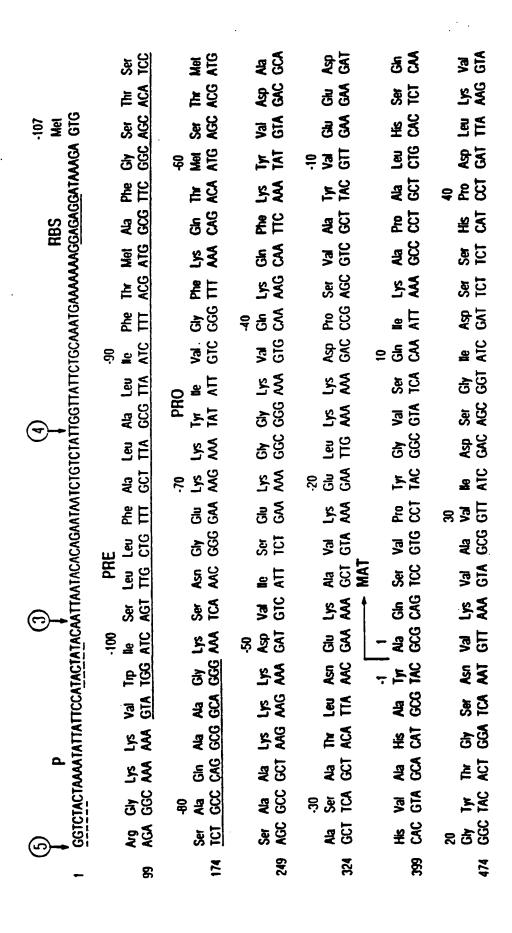


FIG._1B-1

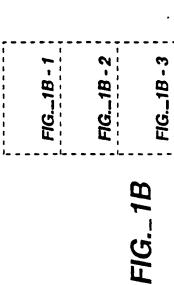
His Gly Thr His Val Ala CAC GGA ACT CAC GTT GCC	Ata 90 Ser Leu Tyr Ata Val Lys TCA CTT TAC GCT GTA AAA		Ala ile Ala Asn Asn Met GCG ATC GCA AAC AAT ATG	Ala lie Ala Asn Asn GCG ATC GCA AAC AAT 140 Val Asp Lys Ala Val GTT GAT AAA GCC GTT	Ala lie Ala Asn Asn GCG ATC GCA AAC AAT 140 Val Asp Lys Ala Val GTT GAT AAA GCC GTT Thy Val Gly Tyr Pro ACA GTG GGC TAC CCT	Ala lle Ala Asn Asn GCG ATC GCA AAC AAT 140 Val Asp Lys Ala Val GTT GAT AAA GCC GTT Thr Val Gly Tyr Pro ACA GTG GGC TAC CCT 190 Phe Ser Ser Val Gly TTC TCA AGC GTA GGA	Ala lie Ala Asn Asn GCG ATC GCA AAC AAT 140 Val Asp Lys Ala Val GTT GAT AAA GCC GTT ACA GTG GGC TAC CCT 190 Phe Ser Ser Val Gly TTC TCA AGC GTA GGA TTC TCA AGC GTA GGA TAC GGG GCG TAC AAC TAC GGG GCG TAC AAC
Ser His	Ser Ala Ala Ser GCA TCA	Tip Ala TGG GCG		Ala Val GCA GTT	Ata Val GCA GTT Ser Thr AGC ACA	Ala Val GCA GTT Ser Thr AGC ACA Ser Phe TCT TTC	Ata Val GCA GTT Ser Thr AGC ACA TCT TTC Lys Tyr AAA TAC
NSP ASP ASP CAC AAC AAC	Ala Pro Ser GCG CCA AGC	110 Gly lle Glu GGA ATC GAG		کې کې	Lys AAA Ser AGC	Lys AAA AGC AGA	Lys AAA Ser AGC AGC AGA Gly GGA
Phe Gin TTC CAA	Gly Val GGC GTT	lle Asn ATT AAC		Ala Ala GCT GCT	Ala Ala GCT GCT Ser Thr Thr Ser : ACT TCC	Ala Ala GCT GCT Ser Thr Thr Ser ACT TCC Ser Asn AGC AAC	Ala Ala GCT GCT Ser Thr Thr Ser ACT TCC AGC AAC Thr Leu ACG CTT
F10 ASN F10 PTO A AAT CCT	Val Leu IT GTA TTA	r TIP Ne IC TGG ATC) r Gly Ser T GGT TCT	Gr Gr GA	Giv Giv GAA GAA GAG	Giv Giv GAA GAA CAA
r Glu Thr :T GAA ACA	80 r lle Gly A ATC GGT	A TAC AGC		130 7 Pro Ser 5A CCT TCT	Pro CCT Gly GGT	Pro CCT GGy GGT GCT	Pro CCT Giy GGT GCT TCT
Val Pro Ser GTT CCT TCT	Asn Asn Ser AAT AAC TCA	Ser Gly Gln TCC GCC CAA		Leu Gly Gly CTC GGC GGA	Giy GGC GCA GCA	Gly GGC Ala GCA GTA	Giy GGC GGC GGC GGC
50 Ser Met Va AGC ATG G	Leu CTT	Ala 100 Asp Gly Se GAC GGT TC		Ser AGC	Ser AGC 150 Val GTT	Ser AGC 150 Val GTT ATT	Ser AGC 150 Val GTT GTT ATT 200 Ala GCA
Gly Ala S GGA GCC A	Ala GCG	Asp A Gly Ala A GGT GCT G		Asn	Asn AAC Val GTA	Asn AAC Val GTA Ser	Asn AAC Val GTA GTC GTC
Ala Gly GCA GGC	ACA Th	Val Leu (GTT CTC (Val GTT	Val GTT GGC	Val GTT GGC GGC 174	Val GTT GGV GGC GGC CTT
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TW TAC	371100
Pre TC	TERM TAAAAAACGGCCTTGGCCCCGCCGGTTTTTATTTTTCTTCCTCCGCATGTTCAATCCGCTCC
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1316 ATAATCGACGGATGGCTCCCTCTGAAAATTTTAACGAGAAACGGCGGGTTGACCCGGCTCAGTCCCGTAACGGCCAAGTCCTGAAACGTCTCAATCGCCG

FIG._1B-3



CONSERVED RESIDUES IN SUBTILISINS FROM

BACILLUS AMYLOLIQUEFACIENS 20 10 AQSVP.G...APA.H..G 30 .TGS.VKVAV.D.G...HP 41 DL...GGAS.VP... 61 . N . H G T H V A G T . A A L N N S I G V L G V A P S A . L Y A V K V L G A . G SG..S.L..G.EWA.N... 130 V.N.SLG.PS.S...A.. 150 160 141 G V . V V A A . G N . G . . . 180 161 Y P . . Y A V G A . 200 190 181 D. N. ASPS. G. LD. A 210 220 201 PGV..QST.PG..Y...NGT 221 SMA.PHVAGAAAL...K... 250 260 241 W...Q.R..L.NT...LG..

FIG._2

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B.amyloliquefaciens B.subtilis B.licheniformis B.lentus

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